The Bacteriome and Metabolome of Human Breast Milk and their association with infant growth

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DECLARATION

I hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work or any part thereof is being, has been, or is to be submitted for another degree in this or any other university. The research described here was carried out in the Division of Medical Microbiology, Faculty of Health Sciences, University of Cape Town under the supervision of Dr Elloise du Toit and Professor Mark Nicol.

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DECLARATION: INCLUSION OF PUBLICATIONS

I confirm that I have been granted permission by the University of Cape Town's Doctoral Degrees Board to include the following publications in my PhD thesis, and where co-authorships are involved, my co-authors have agreed that I may include the publications:

i. Ojo-Okunola A, Nicol M, Du Toit E. Human Breast Milk Bacteriome in Health and Disease. Nutrients. 2018; 10(11):1643.
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ABSTRACT

Human breast milk is a complex species-specific biological fluid universally known as the optimal post-natal source of nutrition for infants and therefore, recommended by the World Health Organization as the exclusive food for infants in the first six months of life. Despite the importance of human breast milk in infant health, study of its composition, especially the bacteriome (bacterial communities) and metabolome (complete set of metabolites), and their relationship to infant health and growth have not yet been comprehensively characterized. This is particularly true in low- and middle-income countries. We therefore conducted a cross-sectional study, nested within an existing birth cohort, the Drakenstein Child Health Study, to describe the bacteriome and metabolome of human breast milk samples collected between 6-10 weeks postpartum from lactating women living in South Africa. The determinants of these components of human breast milk and their role in infant growth were also investigated.

Four commercial DNA extraction kits were compared for DNA extraction from human breast milk samples. The kit showing the best results, including quality and quantity of DNA, as well as best reproducibility, was chosen for further extractions. Using 16S rRNA gene amplicon next generation sequencing, a reproducible bioinformatics sequencing pipeline, and robust multivariate statistical analysis, we confirmed the presence of a diverse bacterial community in human breast milk and identified a core bacteriome, present in 80% of the samples. The bacteriome was shown to cluster into three different profile groups (biotypes) according to the predominant bacterial genus present. Bacterial interactions were suggested by the finding of positive correlations between the relative abundances of bacteria usually found in the oral or skin microbiota. Apart from study site (a proxy for ethnicity in this study), infant birth length and maternal age, no other associations were found between potential socio-demographic and psychosocial determinants and the composition of the human breast milk bacteriome.

Using Nuclear Magnetic Resonance spectroscopy, we quantified forty-nine metabolites in all human breast milk samples. A subset of women with low levels of lactose concentrations were identified. Low lactose was associated with an increase in metabolites associated with mixed acid fermentation and microbial dysbiosis (staphylococcal-predominant biotype). Low-lactose (vs normal lactose) human breast milk correlated with a reduced median duration of exclusive breastfeeding and reduced infant growth (reduced weight and length z-scores) during the period of exclusive breastfeeding. These results suggest that bacterial fermentation of lactose results in low-lactose breast milk, which in turn impacts on breastfeeding outcome.

Taken together, the results presented in this thesis provide a better understanding of human breast milk composition among lactating mothers living in South Africa, their potential determinants and their role in infant growth. Knowledge about the composition of human breast milk may provide

opportunity for diagnostic and therapeutic interventions and help promote (exclusive) breastfeeding for the recommended period to improve infant health.

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ABBREVIATIONS

ANOVA	analysis of variance
BF	breastfeeding
bp	base pair
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
CI	confidence Interval
EBF	exclusive breastfeeding
e.g.	exempli gratia
Etc.	et cetera
GC-MS	gas chromatography- mass spectrometry
HBM	human breast milk
HMO	human milk oligosaccharides
kDa	kilodalton
LC-MS	liquid chromatography- mass spectrometry
mins	minute(s)
mM	millimolar
NCBI	national center for biotechnology information
NGS	next generation sequencing
OTU	operational taxonomic unit
PCA	principal component analysis
PCR	polymerase chain reaction
PERMANOVA	permutational multivariate analysis of variance
pM	picomolar
RNA	ribonucleic acid
rRNA	ribosomal RNA
rpm	revolutions per minute
RT	room temperature
SCFA	short chain fatty acids
sec	second(s)
SM	skim milk
TAE	tris-acetate-EDTA
Treg	T regulatory
Tris-HCl	tris-hydrochloride
UCT	university of cape town
µl	microliter

V	volts
WHO	world health organization
WM	whole milk
%	percent
^{o}C	degree(s) Celsius

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Background and Rationale

Infant breast feeding (BF) emerged as an evolutionary strategy subsequent to the divergence of mammals millions of years ago and natural selection has made milk uniquely suited to nourish infants of each species (1). Human breast milk (HBM) is uniquely adapted and is universally regarded as the optimal post-natal source of nutrition for the growing infant. Breast-fed infants have been shown to have a lower morbidity and mortality during 1st year of life, and reduced risk of respiratory and gastrointestinal infections (2, 3). The components of HBM including water, protein, fatty acids, microbes, immunological factors, glyco-conjugates and metabolites all play important roles in the beneficial properties of BF.

Although analysis of HBM was not included in Human Microbiome Project (HMP), a project designed to comprehensively characterize the presence and function of microbiome colonizing different body sites, several studies have shown that HBM harbours its own microbiome (4-7). The term microbiome was first mentioned in 2001 by Joshua Lederberg as a descendant of the word 'genome' and was described as "the ecological community of commensal. symbiotic, and pathogenic microorganisms that literally share our body space" (8). These micro-organisms include the bacteria, archaea, fungi, lower and higher eukaryotes, and viruses (9). The microbiome has, more recently, been defined as "the entire habitat, including the microorganisms, their genomes and the surrounding environmental conditions" (10), while the collection of all bacteria residing in or on humans (i.e. the bacterial component of the human microbiome) is referred to as the bacteriome (8, 11).

Recently, next-generation sequencing (NGS) has been adopted for microbial identification by profiling of 16S rRNA gene amplicons or whole metagenome shotgun sequencing. The bacterial diversity in HBM has been shown to be larger than originally thought, with more than 600 Operational Taxonomic Units (OTU)s having been identified (4, 7). NGS has also been used to analyse the temporal stability of the bacterial community in HBM, and various factors that lead to variability in HBM bacterial profiles between women have been identified (4).

HBM bacteria are one of the main sources of microbes to the neonate postnatally and may play an important role in 'shaping' the intestinal microbiome early in life including priming and maturation

of the infant's gut immune system (7, 12). Breast-fed infants have been reported to have a decreased faecal species richness with less colonization by bacteria known to be pathogenic, compared with formula-fed infants (13, 14). Recent reports suggest that early microbial colonization has an important role in promoting health and may contribute to reducing the risk of diseases (15-17).

In addition to microbes in HBM, there are numerous nutritive and non-nutritive bioactive factors that elicit protective and promoting effects during the early infant years (13). One of such components is the metabolome defined as "complete complement of all small-molecule (<1500 Da) metabolites found in a specific cell, organ or organism" (18). In HBM, human milk oligosaccharides (HMOs), lactose, lipids, amino acids and biogenic amines are some examples of the metabolites present. A recent study has shown the influence of the HBM metabolome on promoting growth in infants, in part through interaction with the bacteriome (19). For example, fermentation of HMOs by bacteria in the infant's gut produces short chain fatty acids which promote gut epithelial barrier function and modulate immune responses (20, 21).

Despite a complex and diverse bacteriome and metabolome in HBM, previous studies have been limited by sample size with few studies having been performed in sub-Saharan Africa. HBM composition is influenced by a number of maternal, infant, and environmental factors including geographical location, maternal weight and body mass index, maternal health, maternal dietary intake and lactational stage (22-25), though the impact of these variations on the different components of HBM and its health outcomes on the infant is not fully understood. Moreover, the relationship between the HBM metabolome and microbiome has not been well-studied and related to infant health and growth. Thus, a comprehensive analysis of the HBM bacteriome and metabolome is presented in this thesis, including their influence on the breastfeeding outcomes and the health and growth of an infant. We addressed the following questions:

i. What is the composition of HBM bacteriome and metabolome in a cohort of South African mothers enrolled in the Drakenstein Child Health Study?
ii. Do maternal (mode of delivery, maternal BMI, maternal age), infant and environmental factors (smoking, dwelling type, alcohol use) influence the composition of HBM bacteriome and metabolome?

iii. Is there an association between the composition of the HBM bacteriome and metabolome, and breastfeeding outcomes and infant growth in the first year of life?

1.2 Thesis Outline

Chapter 2 is an in-depth literature review into the immunomodulatory, metabolic, and anti-infective role of the HBM bacteriome and its effect on infant health. Key information from the literature was provided by exploring the possible origin of microbial communities in HBM, the bacterial diversity in this niche, the concept of a "core" HBM bacteriome, and the determinants influencing the HBM bacteriome. Lastly, the role of the HBM bacteriome in maternal infectious disease (human immunodeficiency virus infection and mastitis), and cancer was investigated. Key gaps in HBM bacterial research are also identified.

Chapter 3 is a review of the literature regarding the metabolomic diversity of HBM, the technical approaches to studying the HBM metabolome, immune modulatory and anti-infective role of HBM metabolites and their effects on infant health and development. The literature on potential determinants of the HBM metabolome, including maternal infectious diseases such as human immunodeficiency virus infection and mastitis was also reviewed.

Chapter 4 explores the influence of laboratory technical procedures (DNA extraction and milk skimming) on HBM bacterial profiling. This chapter compares four commercial DNA extraction kits and milk skimming in relation to HBM bacterial profiles using ten healthy donor breast milk samples. The kits were evaluated based on their ability to extract high quantities of pure DNA from HBM, and how well they extracted DNA from bacterial communities present in a commercial mock microbial community standard spiked into HBM. Finally, the kits were evaluated by assessing their extraction reproducibility. Bacterial profiles were assessed using Illumina MiSeq sequencing targeting the V4 region of the 16S rRNA gene.

Chapter 5 describes our findings from 16S rRNA amplicon sequencing information on the bacterial composition of HBM samples in a large cohort of lactating women living in South Africa. It also provides information on possible determinants of the HBM bacterial composition including

maternal, socio-demographic and psychosocial factors. Finally, bacterial interactions were studied using co-occurrence networks.

Chapter 6 describes the metabolic profiles of HBM samples from a large cohort of lactating women living in South Africa, as determined by nuclear magnetic resonance spectroscopy. In particular, the relationship between the HBM metabolome and microbiome and their effect on breastfeeding outcomes and infant health is studied.

Chapter 7 concludes the dissertation by summarizing the important findings and main conclusions of each result chapter, limitation of the technical approaches and study design, and recommendations for further research and planned future work.

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CHAPTER 2

HUMAN BREAST MILK BACTERIOME IN HEALTH AND DISEASE

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A.O.-O. wrote all versions of the manuscript; E.dT. and M.N. supervised the project and reviewed all versions of the manuscript. All authors reviewed and approved the final manuscript.

Abstract

It is well-known that, beyond nutritional components, human breast milk (HBM) contains a wide variety of non-nutritive bio-factors perfectly suited for the growing infant. In the pre-2000 era, HBM was considered sterile and devoid of micro-organisms. Though HBM was not included as part of the human microbiome project launched in 2007, great strides have been made in studying the bacterial diversity of HBM in both a healthy state and diseased state, and in understanding their role in infant health. HBM provides a vast array of beneficial micro-organisms that play a key role in colonizing the infant's mucosal system, including that of the gut. They also have a role in priming the infant's immune system and supporting its maturation. In this review, we provide an in-depth and updated insight into the immunomodulatory, metabolic, and anti-infective role of HBM bacteriome (bacterial community) and its effect on infant health. We also provide key information from the literature by exploring the possible origin of microbial communities in HBM, the bacterial diversity in this niche and the determinants influencing the HBM bacteriome. Lastly, we investigate the role of the HBM bacteriome in maternal infectious disease (human immunodeficiency virus (HIV) and mastitis)), and cancer. Key gaps in HBM bacterial research are also identified.

Keywords: bacteriome; human breast milk; bacterial community; mastitis; human immunodeficiency virus (HIV); cancer

2.1 INTRODUCTION

Human breast milk (HBM) is a complex, specific, physiological fluid universally known as the optimal post-natal source of nutrition for infants (1-3). It consists not only of essential nutrients (vitamins, minerals, protein), cells, hormones, immunological and immunomodulatory factors (cytokines, immunoglobulin A, microRNAs), but also of non-nutritive bio-molecules (glyco-conjugates, oligosaccharides) (4) and a vast array of microbes (the bacteria, archaea, viruses, protozoa and anaerobic fungi) known as the human milk microbiota (5-7).

2.2 Human Breast Milk Bacteriome

The presence of bacteria in HBM as both an evolutionary strategy subsequent to the divergence of mammals millions of years ago and an effect of natural selection has made it uniquely suited to nourish infants (8). For example, an infant consuming an average of 800 mL HBM per day has been reported to ingest 10^4–10^6 commensal bacteria (9). These bacteria are not merely present or transient but are rather transcriptionally active and functioning participants in the infant's gut community (10). These bacteria serve as a physiological and continuous source of commensal and potential probiotic bacteria to the infant's gut (2, 11, 12). In addition to the role of HBM bacteria in infants, these bacteria also help in maintaining the mother's health, i.e., aiding in the prevention of mastitis (13).

2.3 Bacterial Diversity in Human Breast Milk

In the pre-2000 era, HBM was considered sterile and devoid of micro-organisms (2). However, in 2003, Martin et al. described the presence of commensal and probiotic bacteria in HBM. The study used culture-dependent techniques and found, in all samples, a predominance of the lactic acid bacteria *Lactobacillus gasseri* and *Lactobacillus fermentum* (14). Lactic acid bacteria, including species of genera *Lactobacillus* and *Bifidobacterium*, are of interest in matters of human health. They are known to limit the growth of potential pathogenic organisms in the gastrointestinal tract due to their ability to produce acetate and lactate from the metabolism of ingested sugars. *Bifidobacterium* was not initially regarded as a typical lactic acid bacteria due to their unrelated genetic structure, however, their habitat overlaps with that of lactic acid bacteria and they produce lactic acid as an end-product of fermentation (15). According to FAO/WHO, select lactic acid bacteria strains with proven probiotic properties are thereby referred to as probiotics (16).

These early descriptions of bacterial diversity in HBM came from utilizing culture-dependent techniques which allowed for the detection of facultative anaerobic bacteria, their close-relatives Gram-positive bacteria and lactic acid bacteria in aseptically collected HBM (9, 14). The more fastidious organisms, such as strict anaerobes which require a more exacting culture media and stricter growth requirements, were not detected (11). More recently, and only using culture-

independent DNA-based techniques including denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) and next generation sequencing (NGS), additional bacterial genera have been detected. These include the obligate anaerobes, particularly *Bifidobacterium* spp., *Bacteroides* spp., and members of the Clostridia class (5, 17).

In the first NGS study of HBM samples, the diversity of bacterial communities, or bacteriomes, was characterized using 454 pyrosequencing to target the 16S rRNA gene. The most abundant genera were found to be *Streptococcus*, *Staphylococcus*, *Serratia* and *Corynebacterium* (5). In another study by Jost et al. (2013), NGS revealed gut anaerobes including Clostridia whose members produce the metabolite butyrate which helps maintain colon health (11). The bacterial diversity of HBM over the course of lactation (colostrum, transitional and mature milk) was also characterized using same technique (18). Several micro-organisms including *Streptococcus* spp., *Staphylococcus* spp. and lactic acid bacteria (*Weisella* spp. and *Leuconostoc* spp.) were found throughout; mature milk samples, however, possessed additional bacterial genera that typically dwell in the oral cavity (18). Similar bacterial diversity patterns were seen using NGS with Illumina MiSeq (19), as well as in two studies using metagenomic approaches (6, 13). While 16S amplicon approaches target bacteria, metagenomic studies allow for the detection of other microorganisms such as fungi, protozoa, archaea and viruses. Another advantage of metagenomic methods is that they allow for taxonomic identification at the species level, whereas 16S can only confidently identify organisms until the genus level. Firmicutes and Proteobacteria were the dominant phyla observed in both the metagenomic studies and the 16S sequencing studies. At the genus level, however, the relative abundance of *Streptococcus* and *Staphylococcus* was relatively low (6, 13). A systematic review of the HBM bacteriome using culture-independent techniques has revealed that these two genera (*Staphylococcus* and *Streptococcus*) may be universally predominant regardless of differences in geographical area or methodological approach (20).

2.4 The Core Milk Bacteriome?

Hunt and co-workers (2011) suggested that there is a “core” HBM bacteriome of nine bacterial genera including *Staphylococcus*, *Streptococcus*, *Serratia*, *Pseudomonas*, *Corynebacterium*, *Ralstonia*, *Propionibacterium*, *Sphingomonas*, and *Bradyrhizobium*. The operational taxonomic units (OTU) were found to represent about half of the observed microbial community, though their relative abundances varied quite significantly among women (5). Since then, various studies have confirmed the hypothesis of a core bacteriome (13, 19, 21). This “core” was not observed across colostrum samples, suggesting that the acquisition of a stable microbial profile is gradual (22).

The core bacteriome may consist of species needed for maintaining efficient ecosystem homeostasis whose loss (or gain) may negatively impact the structure and function of other members in the ecosystem (23). Interestingly, however, it is assumed that the core bacteria are less

affected by the environmental factors (diet, obesity, stress) which are known to alter the composition of the other bacteriome (24).

Differences in this "core" bacteriome have been reported across various studies (5, 19, 21). These differences could be a result of the following factors: sample collection methods (electric pump vs. manual expression, skin cleaning vs. decontamination), use of different DNA extraction kits, storage conditions and freeze/thaw cycles of samples, sequencing platforms, possible biases introduced by the use of primers with the amplification of different 16S rRNA gene hypervariable regions and use of different pipelines in analyzing sequence reads (6, 21). Despite these factors, the identified core bacteria genera commonly included *Staphylococcus*, *Streptococcus*, *Lactobacillus and Propionibacterium* (see Figure 2.1). Formal meta-analysis of studies characterizing the core HBM bacteriome in different geographical locations, however, is required.

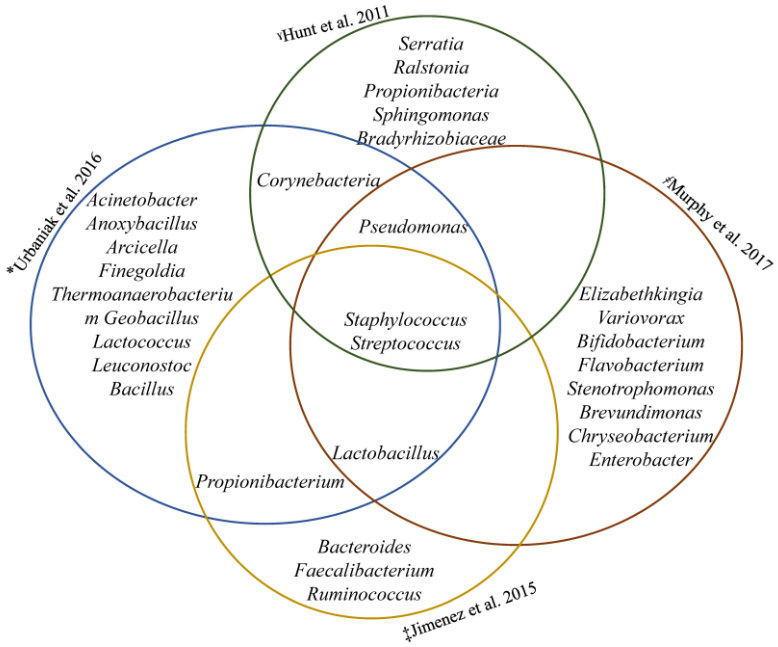

Figure 2. 1*: The core human breast milk bacteriome.* *QIAamp® DNA Stool Kit (Qiagen), V6 region of bacterial 16S rRNA gene, Ion Torrent platform. ᵛQIAmp DNA Stool Mini Kit (Qiagen), V3–V4 region of bacterial 16S rRNA gene, Illumina MiSeq platform. ‡QIAamp DNA Mini Kit (Qiagen) with previous mechanical and enzymatic lysis, V1–V2 region, Pyrosequencing.

2.5 Origin of The Human Breast Milk Bacteriome

There have been several debates about the origin of bacterial communities in HBM. In 2003, Martin et al. used randomly amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) to analyze lactic acid bacteria from HBM, breast skin and areola. It was observed that the lactic acid bacteria isolated from HBM had DNA profiles that were different from those isolated from either the breast skin or the mammary areola (14). Obligate anaerobes (*Bacteroides* spp. and/or

Bifidobacterium spp.) which are unlikely to survive the aerobic conditions of the breast skin have also been isolated from HBM (12, 21).

In addition, an experiment conducted by Hunt et al. (2011) showed that although the bacteriome in the sebaceous skin and HBM share many of the same phylotypes, differences were found. *Streptococcus*, one of the most abundant genera in HBM samples globally, was only a minor component of the sebaceous skin bacteriome. *Propionibacterium*, on the other hand, reported as one of the most abundant in sebaceous skin genera, was not among the most abundant genera found in HBM samples (5). Bacteriome found in HBM using Illumina MiSeq were distinct from the areolar skin in both composition and diversity (25).

2.5.1 Retrograde Flow

It is possible that some bacteria found in HBM come from the transfer of oral and skin bacteria which enter the mammary ducts during suckling in a process called retrograde flow (26). This hypothesis was investigated by Ramsay and colleagues who used ultrasound imaging to demonstrate that there is a high degree of retrograde flow of milk from the infants' mouth back into the mammary ducts during breastfeeding, providing an ideal route for the exchange of bacteria back into the mammary ducts (26). *Streptococcus*, one of the most abundant bacterial genera in the HBM bacteriome, also dominates the salivary bacteriome (5, 27) lending support to the retrograde flow mechanism, however, investigation into whether both bacterial communities share identical species and strains of *Streptococcus* spp. is warranted.

2.5.2 Gut–Mammary-Axis

Another more recent hypothesis on the origin of the HBM bacteriome is the entero-mammary pathway where non-pathogenic, intestinally derived bacteria may be transported to other locations such as mucosal surfaces of the lactating mammary gland through the endogenous cellular pathway known as the mononuclear cells (2, 3, 28).

The translocation of the gut bacteria to the mammary glands is aided by the physiological and hormonal changes during late pregnancy and the increased permeability of the intestinal epithelial lining (27). In support of this hypothesis, animal studies have shown increased bacterial translocation of both aerobic and anaerobic organisms from the gut to the mesenteric lymph nodes and mammary glands in pregnant and lactating mice (28).

In addition, Zhou et al. have found similar bacterial signatures in the dendritic cells (DC), breast milk (BM), intestines and lymph nodes of lactating mice (29) suggesting translocation of bacteria from the intestines by the DC, into the lymphatic system and carried through to the mammary gland environment.

2.5.3 *Mammary Gland Bacteriome*

The human breast tissue bacteriome has recently been determined (30, 31) from breast tissue biopsies collected from different sites within the breast. The viability of the bacteria was confirmed by culture. As in HBM bacteriome, the principal phylum, Proteobacteria, was the major phylum detected in human breast tissue bacteriome. Moreover, the two microbial communities share several bacteria genera (6, 21).

The breast ductal bacteriome has recently been described by analyzing nipple aspirate fluid (NAF) (32). NAF is regularly secreted by the epithelial cells lining the breast ducts that can be collected non-invasively from the duct in most women by applying negative pressure with a syringe attached to a suction cup (33). The duct is in constant communication with the external environment through the areola. There is likely to be interaction of microbes between these various compartments in the mammary environment.

Together, we may view the ecological niches in the human bacteriome, not as isolated environments, but as a network of inter-related communities experiencing constant exchange (5). It seems likely that the HBM bacteriome may be constantly influenced by exposure to other microbial populations associated with mother and child.

2.6 Factors Which May Affect the Human Breast Milk Bacteriome

Many factors have been identified to contribute to the variability of the HBM bacterial community between different women and within the same woman while she is experiencing different physiological, hormonal and pathological conditions. Both maternal and infant factors have been shown to contribute to this variation. While factors such as infant gender have been shown to have no influence (19), studies have shown that maternal health and geographical location play a major role (see Table 2.1).

1. Mode of delivery: An estimate by qPCR (34, 35) claims that women who delivered via caesarean section (CS) have been shown to have a lower abundance of *Lactobacillus* spp. *(L. fermentum* and *L. salivarius)*, *Bifidobacterium* spp. when compared with the higher bacterial counts of women who delivered vaginally. The HBM of mothers who had elective CS also showed decreased members of the family Leuconostocaceae and increased Carnobacteriaceae, when compared with women who delivered vaginally (18). However, in a study by Urbaniak and colleagues which utilized a more robust statistical analysis (19), no difference in bacterial profiles was observed between women who delivered vaginally and those who delivered via emergency CS. It was suggested that this could be due to the initiation of the labor process, including physiological stress and hormonal signals which may influence increased permeability of intestinal epithelial lining for translocation (18, 19).

2. Maternal weight: Higher levels of *Staphylococcus* spp. and lower levels of *Bifidobacterium* spp. were observed in HBM from overweight mothers as compared with normal-weight mothers (36); and a less diverse bacterial community has also been observed in obese mothers (18). This may be due to the metabolic capacity of the bacteriome of obese individuals which has an increased capacity to harvest energy from diet (37).

3. Antibiotics and Chemotherapy: A lower abundance of lactobacilli and bifidobacteria was detected in HBM of mothers who were exposed to antibiotics during the perinatal stage (35). Exposure to anti-cancer chemotherapy also resulted in a reduction of the genera *Bifidobacterium, Eubacterium, Staphylococcus and Cloacibacterium* (38).

4. Maternal health: Gronlund et al. (2007) described that the bacteriome is influenced by maternal health. In his study using direct PCR analysis, allergic women exhibited a significantly lower *Bifidobacterium* spp. in their BM, with their infants also having lower fecal bifidobacteria counts (39). African women with HIV–RNA in their HBM had an increased bacterial diversity and higher abundance of *Lactobacillus* spp. compared to controls (40). Lower abundance of *Bifidobacterium* spp. and *Bacteroides fragilis* group have been detected in HBM of women with celiac disease (41).

5. Lactation stage: A higher bacterial diversity but lower total bacterial count and less bifidobacteria species were detected in colostrum when compared with mature HBM (18). *Bifidobacterium* spp. and *Enterococcus* spp. counts, along with total bacteria increased as the lactation stage progressed (34). In a similar study, however, transition milk samples were observed to possess higher diversity than colostrum and mature milk (7, 34).

6. Geographical location: The bacterial genera found in HBM of Spanish mothers were different to those of Americans (5, 7), or Finnish women (18) using sequencing techniques with a similarly high throughput. In a study by Kumar et al., Chinese women had high levels of Actinobacteria in comparison to the similarly high levels of Bacteroidetes detected in Spanish women (42).

7. Gestational age: *Bifidobacterium* spp. were observed to be higher in HBM of women with term babies than in preterm gestation.

Table 2. 1: Factors influencing human breast milk bacteriome.

Factors Influencing Human Breast Milk Bacteriome	Bacteriome	References
Mode of delivery		
Caesarean section	↓ *Bifidobacterium* spp., ↑ Proteobacteria, ↓ *Lactobacillus spp (L. fermentum* and *L salivarius),* ↓ Leuconostocaceae, ↑ Carnobacteriaceae	(18, 34, 35)
Vaginal delivery	↑ *Bifidobacterium* spp., ↑ *Lactobacillus* spp.	(34, 35)
Maternal weight		
Overweight mothers	↑*Staphylococcus,* ↓ *Bifidobacterium*	(36)
Obese mothers	Less diverse bacterial community	(18)
Antibiotic* and *Chemotherapy		
Perinatal antibiotics usage	↓ *Lactobacillus/Bifidobacterium*	(35)
Chemotherapy	↓ *Bifidobacterium,* ↓ *Eubacterium,* ↓ *Staphylococcus* and ↓ *Cloacibacterium*	(38)
Maternal health		
Allergy	↓ *Bifidobacterium*	(39)
Celiac disease	↓ *Bifidobacterium* spp., ↓ *Bacteroides fragilis* group	(41)
HIV	↑ Bacterial diversity, ↑ *Lactobacillus* spp.	(40)
Geographical location		
Spanish women	↑ Bacteroidetes	(42)
Chinese women	↑ Actinobacteria	(42)
Lactation stage		
Colostrum	↑ Bacterial diversity, ↓ *Bifidobacterium* spp., ↓ Total bacterial count	(18)
Transition milk	↑ *Bifidobacterium,* ↑ *Enterococcus* spp. counts, ↑ Total bacteria count	(7, 34)

HIV: human immunodeficiency virus, ↑ and ↓ corresponds to increase and decrease levels of specific bacteria genera respectively.

2.7 Plausible Functions of Human Breast Milk Bacteria

The HBM bacterial communities play a role in reduction of the incidence and severity of infections in the breastfed infant via mechanisms such as competitive exclusion and production of antimicrobial compounds. HBM bacteriome also improve the intestinal barrier function by increasing mucin production and reducing intestinal permeability (2, 3).

2.7.1 Vertical Transmission and Seeding of Infant Gut by HBM Bacteria

Evidence for vertical transmission of maternal bacteria, via milk, to the infant's gut has been shown in humans (12, 14). *Lactobacillus* spp. sequences isolated from infant feces showed identical

patterns to those found in their respective maternal HBM but differed from the profiles found in the maternal vagina (14). Identical bacterial strains of bifidobacterium (*Bifidobacterium breve* and *Bifidobacterium longum* subsp. longum), and *Lactobacillus plantarum* have been confirmed in HBM and infant feces of mother-infant pairs, suggesting vertical transfer from the mother's milk to the infant (21, 43, 44).

Recently, additional supporting evidence for vertical transmission of maternal microbes has been published. Shotgun metagenomics was used to demonstrate the transfer of specific strains of *Bifidobacterium* spp., *Ruminococcus bromii*, and *Coprococcus comes* within different mother-infant pairs (10). Another study compared the fecal bacteriome of breastfed infants, whose gut is dominated by *Bifidobacterium* spp. and *Lactobacillus* spp. transmitted from HBM, to that of formula-fed infants, whose gut is predominantly colonized by enterococci, enterobacteria and *Clostridium difficile*—a pathogen associated with enteric and atopic diseases (45, 46).

2.7.2 Anti-Infective Activities of HBM Bacteria

In vitro studies show that *Lactobacillus rhamnosus* and *Lactobacillus crispatus*, isolated from HBM have anti-infective properties against *Staphylococcus aureus (S. aureus). S. aureus* has been implicated in mastitis (9), antibiotic-resistant nosocomial infections, and neonatal infections. HBM-derived lactobacilli strains, particularly *Lactobacillus salivarius (L. salivarius)* CECT5713, produce not only in vitro antibacterial activity, but also a protective effect against *Salmonella enterica* serovar Cholerasuis *(S. enterica)* CECT4155 in animal models. This is mediated through inhibiting the adhesion of *S. enterica* to mucins and increasing the likelihood of survival of infected mice (47).

Additionally, HBM lactic acid bacteria protect the physiological environment of the gut through mechanisms such as the production of organic acids and the lowering of pH to inhibit the growth of various facultative and anaerobic bacteria (47, 48).

2.7.3 Immuno-Modulatory Activities of HBM Bacteria

HBM bacteria provide a source of bifidobacteria to the infants' gut. Bifidobacteria in turn activate T-regulatory cells which can result in improved resistance to pathogenic microorganisms (2, 45). In addition, the human milk metagenome has been shown to contain immunomodulatory DNA motifs which may help decrease exaggerated inflammatory responses to colonizing bacteria (6). HBM derived probiotic strains, *Lactobacillus fermentum* CECT5716 and *Lactobacillus salivarius* CECT5713, have demonstrated in vitro immunomodulatory activity by modulating the activation of peripheral blood mononuclear cell (PBMC) subsets, CD8+ natural killer cells, Treg cells, and several cytokines and chemokines. This effect was not seen with probiotic bacteria of non-milk origin (49).

2.7.4 Anti-Allergic Properties of HBM Bacteria

A beneficial association has been proposed between HBM lactic acid bacteria and allergies. For example, animal studies have shown that probiotic lactobacilli (of HBM origin), *Lactobacillus gasseri* together with *Lactobacillus coryniformis*, decrease the occurrence and severity of allergic responses to cow milk protein (50). Although a randomized, controlled trial showed that probiotic supplementation in the first six months of life did not reduce the risk of atopic eczema (51), other studies found that supplementation with specific *Lactobacillus* spp. and/or *Bifidobacterium* spp. in mothers led to a reduced infant eczema at one year and two years of age (52, 53). The Hygiene Hypothesis suggests that the anti-allergy properties of probiotics are due to the down-regulation in the production of Th2 cytokines by inducing a Th1 response (50). Disturbance in the regulation of the immune system is considered an underlying cause of allergies (54).

It is also interesting to note that the presence of viridans streptococci, one of the dominant bacterial groups in HBM, seems to be a feature of a healthy infant gut in contrast to that of atopic infants whose gut is dominated by *Klebsiella* spp. (55). Animal studies have shown that defective maturation of the immune system in animals that lack appropriate host–microbe interactions during early life makes them prone to allergic immune responses. This defective immune maturation occurs with the introduction of even a single strain of bacteria such as *Bifidobacterium* spp. during the neonatal phase (56).

2.7.5 Metabolic Activities of HBM Bacteria

HBM bacteria are essential for the digestion of oligosaccharides (the fourth main component of HBM). Infants lack the necessary enzymes to digest these and, instead, the HBM microbes ingested during feedings break them down into short chain fatty acids (SCFA); this end-product also serves as one of the main energy sources for colonocytes (57). This is helpful for increased nutrient absorption as the gut of an infant is much shorter than in adults leading to quick transit of food (7).

2.7.6 Anti-Tumour Properties of HBM Bacteria

HBM bacteriome may have an anti-tumor role. In vitro studies have demonstrated that the heat-killed cells and cytoplasmic fractions of *Enterococcus faecalis* and *Staphylococcus hominis* isolated from HBM possess anti-tumor activity against a breast cancer cell line (58). Another in vitro study has demonstrated the therapeutic, anti-cancer activity of lactic acid bacteria, *Lactococccus lactis* subsp. *lactis*, against colon cancer (59), This is relevant to both mother and infant health.

2.8 Implications of The Human Breast Milk Bacteriome In Disease

2.8.1 Mastitis

Lactational mastitis is an inflammatory condition of the mammary gland that occurs in about 30% of breastfeeding women (60, 61). It is characterized by local symptoms (localized warmness and soreness on the affected breast, pain, redness, swelling of the breast), in addition to systemic symptoms (fever). It is also a major reason breastfeeding (BF) is discontinued (62, 63).

Mastitis is a dysbiosis of the HBM bacteriome characterized by rapid growth of opportunistic pathogenic bacteria including members of *Staphylococcus* and/or *Streptococcus*, Corynebacterium and depletion of commensal bacteria (*Lactococcus* and *Lactobacillus*) (60, 64, 65). In acute mastitis, the bacteria count of *Staphylococcus aureus* (*S. aureus*) is said to increase greatly to about 4.0 log10 cfu/mL from 1.5–3.0 log10 cfu/mL in healthy subjects. Another species, *Staphylococcus epidermidis* (*S. epidermidis*), normally appearing on skin and mucosa, is an under-recognized causative agent of lactational mastitis. This lack of recognition may occur if the clinician believes its presence stems from contamination of HBM samples with skin flora (60), however, recent studies have shown it plays an increasing role (13).

Coagulase-negative staphylococci (CNS) and *S. epidermidis* have been isolated in HBM of women with chronic mastitis. The chronic nature is because *S. epidermidis* forms biofilms and is resistant to many antibiotics (63). Recent studies also show that the HBM metagenome in women with mastitis is different based on the stage/type of infection. While *S. aureus* was the most common etiological agent of acute mastitis, *S. epidermidis* was found to be the most common bacteria in subacute mastitis (13).

A reduced bacterial diversity and species richness has been observed in HBM of women with mastitis (13, 64). Phage related sequences were also observed in HBM of women with acute mastitis. This is because the virulence factors of *S. aureus* are encoded inside phages, making it easier for the bacteria to evade the host's immune system (13).

Patel et al. (2017) observed members of the genus *Staphylococcus* to be differentially abundant in sub-acute mastitis. Genes related to bacterial chemotaxis and invasion of epithelial cells, bacterial motility proteins and secretion system were enriched in women having sub-acute and acute mastitis (64).

A randomized clinical trial has shown that HBM derived probiotics, *Lactobacillus salivarius* CECT5713 and *Lactobacillus gasseri* CECT5714, when compared with antibiotics (65), are a complementary and effective substitute for the treatment and control of mastitis.

2.8.2 Human Immunodeficiency Virus (HIV)

HBM of women infected with HIV has been shown to have a different bacterial composition compared with non-infected women. An increase in both bacterial diversity and *Lactobacillus spp.* frequency has been observed. In contrast, *Staphylococcus hominis* (*S. hominis*) and *S. aureus* were significantly reduced (40) in HIV-infected women. The reduction of these two *Staphylococcus* species may be due to the inhibitory features of lactic acid bacteria against *S. aureus* (40). In addition, a pathological or disturbed HIV-positive immune system could be a reason for the observed results. In a similar study, however, there was no difference either in bacterial diversity or in the bacterial profiles between HIV-positive and HIV-negative women (66). The discrepancies in these results are unknown but could, however, be due to a difference in geological location, methodological differences or the small sample sizes used in the later study.

2.8.3 Cancer

Although there has been no direct study on breast milk bacteriome and (breast) cancer, as women with cancer seldom breastfeed, the microbial ecology of other compartments within the mammary environment such as the breast tissue bacteriome and breast ductal bacteriome (using nipple aspirate fluid) have been linked to cancer (32, 67).

Microbial dysbiosis was implicated in breast cancer in a study by Urbaniak and colleagues. There was a depletion of the lactic acid bacteria, *Lactococcus* spp. and *Streptococcus* spp., and an increased abundance of *Bacillus* spp., *Staphylococcus* spp. and the family Comamonadaceae and Enterobacteria. *Escherichia coli* isolated from breast cancer patients in the same study induced DNA double-stranded breaks (67).

Bacteria may have a systemic influence in the host, promoting, regulating and training the immune system (68). Bacteria maintain the health of breast tissue by stimulating resident immune cells. In dysbiosis, a condition characterized by the reduction of specific bacterial taxa, there may be lower immune cell stimulation by such bacteria. This in turn creates an environment which may be conducive for breast tumor formation. Xuan et al. found that compared with the adjacent normal tissue, there was a significant reduction of *Sphingomonas yanoikuyae* in breast cancer patients (69). In a similar study, a member of the *Sphingomonadaceae* family was enriched in nipple aspirate fluid from healthy controls while *Alistipes* was enriched in breast cancer patients (32).

The mechanisms, e.g., inflammation and DNA damage, through which bacteria play a role in cancer should be studied further, using animal models, as it’s unsure whether the association of specific bacteria with tumors could be due to their ability to thrive well in particular milieus or because they cause cancer (70).

2.9 CONCLUSIONS

In conclusion, the HBM bacteriome has been shown to play a role in anti-infection, immunomodulation and metabolic activity and is known to be influenced by maternal and socio-economic factors. A better understanding of the factors influencing HBM bacteriome may make it possible to manipulate bacterial communities to improve the health and development outcomes of infants. Lastly, the HBM bacteriome may be associated with, or cause, specific disease conditions.

2.9.1 Gaps Identified

1. There are very limited animal studies demonstrating the specific role of breast milk bacteria in disease conditions.
2. There are few integrative studies exploring the interplay between HBM bacteriome and its metabolites, and their collective role in diseased condition.
3. There is insufficient data on some of the identified HBM bacterial groups. It isn't enough to simply characterize the bacterial diversity of HBM; rather, the role that each of the bacterium play in maintaining the microbial ecology of the HBM bacteriome and their role in infant and maternal health must be understood.
4. There are few studies on HBM bacteriome and the factors influencing low socio-economic regions, such as many African settings. These regions also boast less research of infant nutrition and this research is necessary to know if there are any underlying genetic mechanisms involved.

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CHAPTER 3

THE DETERMINANTS OF THE HUMAN BREAST MILK METABOLOME AND ITS ROLE IN INFANT HEALTH

Abstract

Human breast milk is needed for optimal growth as it satisfies both the nutritional and biological needs of an infant. The established relationship between breastfeeding and an infant's health is attributable to the nutritional and non-nutritional, functional components of human breast milk including metabolites such as the lipids, amino acids, biogenic amines and carbohydrates. These components have diverse roles, including protecting the infant against infections and guiding the development of the infant's immature immune system. In this review, we provide an in-depth and updated insight into the immune modulatory and anti-infective role of human breast milk metabolites and their effects on infant health and development. We also review the literature on potential determinants of the human breast milk metabolome, including maternal infectious diseases such as human immunodeficiency virus and mastitis.

Keywords: metabolome; human breast milk; metabolite profiles; human immunodeficiency virus , mastitis.

3.1 INTRODUCTION

Human breast milk (HBM) is the optimal nutrition for infants, and the WHO recommends exclusive breastfeeding (EBF) for the first 6 months of life (1) with continued breastfeeding (BF) up to two years of age. EBF, especially during the first 6 months of life, has a protective effect against acute respiratory tract infection (RTI) and mortality due to diarrheal illness in developing countries (2, 3). In pre-term infants or (very) low birth weight infants, EBF has been linked to a reduction in infant morbidity and mortality in the first year of life (4, 5).

The benefits of BF an infant are long-lasting, and extend beyond the period of BF (6). For example, BF has been shown to be beneficial for cardiovascular health and for the prevention of hypertension and type I and II diabetes during childhood and later adolescent life (7). Also, EBF has been linked to a lower risk of childhood obesity, which is fast becoming a global epidemic (8). It has also been estimated that by increasing the rate of BF in low and middle-income countries (LMIC), 823,000 child deaths per year due to gastrointestinal disorders could be prevented (9).

In full-term infants, HBM is almost always sufficient in providing essential nutrients for infant growth and development, irrespective of the mother's own nutritional status (10). The components of HBM including water, protein, fatty acids, microbes, immunological factors, human milk oligosaccharides and metabolites all play important roles in the beneficial properties of BF.

3.2 Human Breast Milk Metabolome

HBM metabolites are low molecular weight compounds which include the intermediate and end products of metabolism, originating from different metabolic processes in the mammary gland. Some metabolites, such as lactose, are produced in the endoplasmic reticulum of the milk secretory cells (11). Other metabolites are produced through the metabolic processes of resident microbes in HBM or originate from other cell types and are filtered through the mammary epithelium from bloodstream (12, 13). While the abundance of some metabolites, such as fatty acids, is highly variable, the abundance of other metabolites, e.g., urea, is highly conserved suggesting a specific functional role in the infant (13). This review describes the nutritional and non-nutritional biologically active metabolites of HBM, their determinants and their role in infant immune development, gut microbial colonization, and infant health and developmental outcomes.

The HBM metabolome can be investigated using two main technical approaches: proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and mass spectrometry (MS). ^{1}H-NMR spectroscopy has the following advantages: (1) it is a highly reproducible technique that specifically identifies small molecule in a non-targeted, non-destructive manner from biological fluids; (2) it does not require extra steps during sample preparation, such as separation or sample derivatization; (3) it allows for the identification of a wide range of compounds and provides information about chemical structure; (4) it is the preferred method for the detection of hydrophilic and uncharged

compounds, such as sugars, which are often undetectable by liquid chromatography-mass spectrometry (LC–MS) methods (14-16). MS, on the other hand, is (1) highly sensitive, with a detection limit in the nanomolar range; (2) requires only a small volume of sample as its starting material; (3) it is the recommended technique for targeted analysis; (4) when coupled with gas chromatography, it is preferred in the detection of hydrophobic and volatile compounds, such as lipids (17). Overall, both techniques can be used in a complementary manner to maximize the identification of different metabolites within the HBM sample.

The first metabolomic study on HBM was conducted in 2012 by Marincola et al (18) using both NMR spectroscopy and gas chromatography-mass spectrometry (GC-MS) to study the aqueous and lipid fractions, respectively, of preterm HBM. Compared to the metabolites in pre-term formula milk, the aqueous extracts of preterm HBM were shown to have high lactose concentrations. The pre-term formula milk, on the other hand, had a higher concentration of maltose (18). The HBM metabolome was further explored by Practico et al (19) with NMR spectroscopy. Twenty HBM samples were analyzed and a total of 43 metabolites were detected at least once in all HBM samples including amino acids, short chain fatty acids, oligosaccharides, sugars, phenolic compounds, tricarboxylic acid (TCA) cycle intermediates and N-trimethyl moieties (choline and acetyl-L-carnitine) (19).

Using mass spectrometry coupled with both gas chromatography and liquid chromatography, Qian et al (20) confirmed the unique metabolic profile of HBM and highlighted the significant differences between HBM and bovine or formula milk, in relation to both the presence and abundance of most metabolites (20). For example, non-esterified fatty acids such as saturated fatty acids with aliphatic tails <16 carbons (capric acid, myristic acid, lauric acid), mono-unsaturated fatty acids (oleic acid, palmitoleic acid, eicosenoic acid) and essential polyunsaturated fatty acids (PUFAs) such as linoleic acid and α-linolenic acid were highly abundant in HBM compared with bovine or formula milk (20).

3.3 Specific Human Breast Milk (HBM) Metabolites and Their Role in Health

3.3.1 Carbohydrates

3.3.1.1 Lactose

Lactose represents the most abundant metabolite and the major carbohydrate in HBM (21). It is the primary source of calories, providing about 40% of total energy value of HBM to the infant. It is the major osmotic component regulating HBM volume by drawing water into the intracellular secretory vesicles (13, 21). Lactose concentration ranges between 6.7-7.8 g/dl in mature HBM, and

its concentration is the least variable of the macronutrients as it is tightly regulated by the mammary gland (22, 23).

Lactose induces innate immunity by upregulating gastrointestinal antimicrobial peptides (AMPs), as demonstrated in the colonic epithelial cell line T84 and in THP-1 monocytes and macrophages. This induction may promote intestinal homeostasis and protection of the gut against pathogenic micro-organisms (24). In addition, a recent study has demonstrated an antagonistic effect of lactose on growth of specific bacteria. The concentration of lactose in HBM was shown to be negatively correlated with the HBM bacterial genera *Enterobacter* spp. and *Actinomyces* spp. (25) which contain important opportunistic and multi-resistant bacterial species (26, 27).

Lactose concentration has been shown to be positively correlated with the volume of HBM intake and the number of HBM feeds per day (28, 29). Unlike lipids, lactose concentration was shown to be positively associated with infant weight and adiposity gains between 3 and 12 months of life (28). Also, the influence of HBM lactose on infant growth has been confirmed in a computer model of a newborn baby (from birth to age 6 months) which simulates the baby's metabolism of HBM to understand the mechanisms of infant growth (30).

Lactose also forms part of many carbohydrate-based bioactive compounds in HBM, such as forming the backbone of oligosaccharides (10). Lactose aids mineral absorption in infants including calcium, copper, magnesium and manganese (31).

3.3.1.2 Human milk oligosaccharides

HMOs are a group of bioactive compounds representing the third most abundant metabolite in HBM, after lactose and lipids (32). Compared to other mammalian milk, HBM contains a higher variety (>200), and more complex structures of soluble oligosaccharides, of which 162 chemical structures have been characterized (33). Although diverse and structurally complex, each lactating mother synthesizes a unique compositional subset (34).

HMOs are complex sugars with a lactose core at the reducing end, and are differentiated based on the linkages with one or more building blocks: D- glucose (Glc), L- fucose (Fuc), D- galactose (Gal), N-acetylglucosamine (GlcNAc) or N-acetylneuraminic acid (Neu5Ac) residues (32). HMO composition depends, in part, upon the expression of two specific genes and as such, individual women synthesize different sets of oligosaccharides (13). For example, fucose residues may be attached to a lactose core by an α 1, 2-linkage, which is catalyzed by the enzyme, fucosyltransferase 2 (FUT2), encoded by the secretor gene (*Se*) or by means of α 1, 3/4-linkages catalyzed by the enzyme, fucosyltransferase 3 (FUT3), which is encoded by the Lewis gene (*Le*) (Figure 1) (13). Women can therefore be differentiated into being a secretor or a non-secretor depending on the expression of active FUT2 enzymes. As such, women can have either of four different phenotypes

namely Lewis-positive Secretors (Se+Le+), Lewis-negative Secretors (Se+Le–), Lewis-positive non-Secretors (Se–Le+) or Lewis-negative non-Secretors (Se–Le–) depending on the expression of FUT2 and/or FUT3 gene as shown in Figure 3.1 (35). Non-secretors have low or undetectable concentrations of 2'-fucosyllactose (2'-FL), lactodifucotetraose (LDFT), lacto-N-fucopentaose (LNFP) I, or lacto-N-difucohexaose (LNDFHI) I in their HBM which are all α-1,2-linked fucosylated HMOs (13).

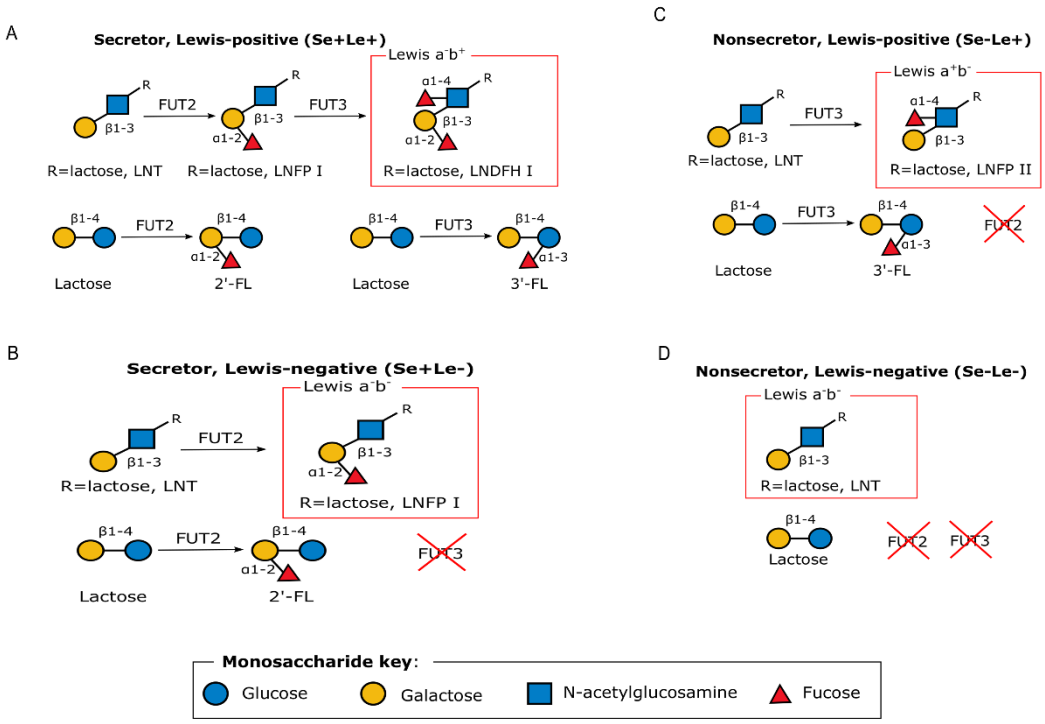

Figure 3. 1: Graphical representation of the enzymatic processes that lead to the different HMO phenotypes. Adapted from Bode 2012 (36). FUT2: Fucosyltransferase 2; FUT3: Fucosyltransferase 3. 2'-FL: 2'-fucosyllactose; 3'-FL: 3'-fucosyllactose

HMOs act as prebiotics which selectively stimulate the function of beneficial bacteria such as *Bifidobacterium* spp. while suppressing the growth of pathogens (37). HMOs are resistant to digestion during their passage through the alimentary canal as human infants lack the glycolytic enzymes needed to break them down. Instead, they are digested by certain commensal bacteria in the infant gut to produce short chain fatty acids (SCFAs) which help in establishing a stable ecosystem in an infant's gut by modulating the immune system and promoting the gut epithelial barrier function (38, 39). SCFAs drive the development and function of regulatory T (Treg) cells thereby limiting intestinal inflammation. They also serve as an energy source for the epithelial cells of the colon and produce an acidic milieu in the gut, making it inhospitable to potentially pathogenic microbes (40, 41). SCFAs such as butyrate, regulate gene expression through inhibition of histone deacetylase. This action may lead to the inhibition of interferon-γ production and suppression of nuclear factor κB (NF-kB) activation in human colonic epithelial cells (42). NF-κB, for example, is

responsible for early immune inflammatory responses and its dysregulation is seen in inflammatory bowel diseases (IBDs) (43).

HMOs inhibit many enteric pathogens, such as toxin-producing *Escherichia coli*, which causes diarrhea in infants (44), *Campylobacter* spp. (45) and Norovirus spp. (46) by acting as a decoy receptor for pathogens. HMOs are structurally related to glycans on the intestinal epithelial cell surfaces, to which pathogenic bacteria bind. Breastfed infants, for example, are protected against *Campylobacter jejuni*, which causes diarrhea, as 2'-fucosyllactosamine found on intestinal cells, which acts as a receptor for *C. jejuni*, is also present on HMOs (38, 47). HMOs have also been shown to reduce attachment of the protozoan parasite, *Entamoeba histolytica* (*E. histolytica*), and associated cell toxicity in a human colon adenocarcinoma cell line. The main adhesion-related virulence factor in *E. histolytica* is a lectin which binds with galactose and N-acetyl-galactosamine, and HMOs containing terminal galactose, such as Lacto-N-tetraose (LNT), act as decoy receptors (48). Cell culture studies have shown that pooled HMOs reduced the invasion and virulence of *Candida albicans* in human premature intestinal epithelial cells (pIECs) by delaying the induction of hyphal morphogenesis after inoculation of *C. albicans* onto pIECs (49).

Different groups of HMOs often exhibit specific properties. For example, sialylated HMOs provide sialic acid needed for neural development, function and cognition (36). Also, a significantly lower concentration of disialyllacto-N-tetraose (DSLNT) has been observed in HBM of mothers whose infants developed necrotizing enterocolitis compared to healthy infants, suggesting a possible protective role of DSLNT to the intestinal tissues of preterm infants and very-low-birth-weight infants (50).

3.3.2 Lipids

Lipids in HBM are complex and diverse and provide 45% - 55% of the total energy needed by infants to support optimal growth (51). Compared to other macronutrients, the lipid composition of HBM is highly variable, and can be affected by duration of breastfeeding, time of day, stage of lactation, maternal nutritional status, and particularly, maternal diet (related to geographic location) (51-53).

Lipids play a role in membrane structure, signal transmission and cell recognition in signaling pathways, lipoprotein metabolism and as a source and carrier of lipid-soluble vitamins (54). For example, palmitic acid, which is a saturated fatty acid, is not only an energy source for the infant but also acts as a pulmonary surfactant that reduces the surface tension at the alveolar air-liquid interface and prevents collapse of alveoli (55). Triacylglycerides are the most abundant lipids in HBM. The remaining lipid classes include diacylglycerides, monoacylglycerides, cholesterol, phospholipids (phosphatidylinositol, phosphatidylserine, sphingomyelin, phosphatidylcholine,

phosphatidylethanolamine), and free fatty acids (Table 3.1) (53). Phospholipids play a vital role in the infant's immune and inflammatory responses, while sphingomyelin plays a role in central nervous system myelination (56). In a randomized control trial, low birth weight infants randomized to receive sphingomyelin-fortified milk showed improved neurobehavioral development during infancy compared with the control group which received only milk (57).

Table 3. 1: Lipid classes in human breast milk. Adapted from (53).

Lipid classes	**Proportion of total lipids in HBM (%)**
Triacylglycerides	98.1–98.8
Phospholipids	0.26–0.8
Phosphatidylinositol	ND
Phosphatidylserine	ND
Sphingomyelin	ND
Phosphatidylcholine	ND
Phosphatidylethanolamine	ND
Cholesterol	0.25–0.34
Non-esterified fatty acids (free fatty acids)	0.08–0.4
Diacylglycerides	0.01–0.7
Monoacylglycerides	Traces

ND: Not determined

All these components make up the milk fat lipid globules (MFLG) which are produced by the alveolar cells of the mammary gland. The MFLG consists of a core of triacylglycerides and a membrane of phospholipids (58). Triacylglycerides are molecules comprised of mixtures of three fatty acids esterified to a glycerol backbone. Triglyceride synthesis involves specific positioning of the fatty acids at the outer sn-1 and sn-3, and center sn-2 positions of the glycerol molecule (59).

Palmitic acid with a 16-carbon backbone (16:0) is the most common saturated fatty acid in HBM and has a preferential positioning of its fatty acids at the sn-2 position, instead of the sn-1,3 positions that are typical of human tissue lipids, infant formula and vegetable oils common in human diets (59). Studies have shown that this specific position of triglycerides in palmitic acid improves the absorption of both palmitic acid and macroelements such as calcium and magnesium. This improved absorption decreases constipation and enhances the BF infant's intestinal well-being (59, 60).

Lipid fractions in HBM include ~34% to 47% saturated fatty acid with mainly ~17%–25% of palmitic acid, ~31% to 43% monounsaturated fatty acids, ~ 12% to 26% n-6 polyunsaturated fatty acids (PUFA), and ~ 0.8% - 3.6% of n-3 PUFA. PUFA are fatty acids containing two or more double bonds along their carbon backbones and include two biologically important subgroups in

HBM, the n-3 and n-6 essential fatty acids represented by α-linolenic acid (ALA) and linoleic acid (LA) respectively. Both ALA and LA are needed for the growth and maturation of various organs in the infant, especially the brain and eye (61). Although PUFA have been shown to prevent various allergic diseases in several studies, recent systematic reviews concluded that there is insufficient evidence that HBM PUFA influence the risk of childhood allergic and respiratory outcomes (62, 63).

ALA can be converted to the long chain-polyunsaturated fatty acids (LC-PUFA), eicosapentaenoic acid (EPA) and docohexaenoic acid (DHA) while LA is converted to arachidonic acid (AA) through consecutive steps involving desaturation and elongation. LC-PUFA of the n-3 and n-6 series are indispensable nutrients with anti-inflammatory and inflammatory activities respectively (62). Table 3.2 below summarizes the major LC-PUFA in HBM, their source and functions.

Table 3. 2: Long chain-polyunsaturated fatty acids (LC-PUFA) in HBM.

Long chain-polyunsaturated fatty acids (LC-PUFA)	**Source, functions and determinants**	**References**
n-3 series		
Docohexaenoic acid (DHA)	Higher DHA concentration in HBM leads to improved neurodevelopmental and vision outcomes in infants. DHA concentration is greatly influenced by dietary intake especially of fish or fish oil supplements in lactating mothers. A dose dependent relationship exists between maternal DHA intake and its concentration in HBM, as an increased DHA concentration in HBM has been observed in lactating women supplemented with DHA. A lower AA to DHA ratio is found in HBM of women from Asian and Scandinavian countries due to higher feeding of DHA-rich fish.	(51, 53, 61, 64, 65)
Eicosapentaenoic acid (EPA)	EPA competes with AA for 5-lipoxygenase and cyclooxygenase enzymes needed for the metabolism of AA, thereby antagonizing the pro-inflammatory effects of AA. Lower concentrations of EPA and DHA in HBM have been associated with allergy in children.	(66, 67)
n-6 series		
Arachidonic acid (AA)	AA is the most abundant of the PUFA with ~0.5% of total fatty acids in HBM. AA content of HBM is relatively stable among women despite variations in diet and lifestyles as it is derived from pre-existing maternal stores. AA serves as a progenitor to signaling molecules, leukotrienes (LTs), thromboxane and the prostaglandins. These products have inflammatory and atherogenic effects	(53, 66, 67)

	on cells. High AA:EPA ratio in HBM has been associated with the development of allergy symptoms at 18 months.	

3.3.3 Biogenic amines

Biogenic amines are low molecular weight nitrogenous organic bases that are secreted in HBM. They include the polyamines (spermine, spermidine and putrescine), together with the monoamines (tyramine) and diamines (histamine and cadaverine) (68). The polyamines are the main biogenic amines found in HBM, with the most abundant being spermine and spermidine. Polyamines are synthesized from the precursor, ornithine, through the action of the enzyme, ornithine decarboxylase. HBM is the first source of exogenous polyamines for the growing infant (69). Polyamines inhibit cytokine synthesis in human mononuclear cells, downregulating the pro-inflammatory cytokine response (70). Polyamines are involved in cell growth and proliferation (68, 71). Rapidly growing tissues, such as the mucosa of an infant's gut require high concentrations of polyamines (69).

A higher polyamine concentration has been observed in HBM of women with preterm babies compared to those with full term babies in early lactation (69), suggesting a greater requirement of this metabolite for the undeveloped intestinal tissues of preterm babies.

A qualitative case study and mathematical model which evaluated the correlation between mean spermine concentration of HBM in the first month of life and allergy appearance estimated that at least 5.02 nmol/ml of spermine is needed to prevent the appearance of allergy in breastfed infants (72). Another study showed that putrescine and spermine concentrations were lower in mature HBM from atopic mothers than non-atopic mothers (73).

3.3.4 Nonprotein Nitrogen Molecules

HBM nonprotein nitrogen comprises many bioactive molecules including the nucleotides, free amino acids, amino sugars, creatine, urea, carnitine and nucleic acids. These make up approximately 25% of the total nitrogen in HBM (56, 74, 75).

3.3.4.1 Free amino acids

Free amino acids including alanine, glutamate, glutamine, isoleucine, threonine and valine, are more readily absorbed than protein-derived amino acids and they provide a nitrogen source for growing infants (76). Free amino acids are also known to give HBM its unique taste as each free amino acid has its own flavor and can be sensed by receptors in the taste-goblet (20).

Glutamine is the most abundant free amino acid and supplies ketoglutaric acid for the citric acid cycle. It also serves as a major energy substrate for enterocytes and as a brain neurotransmitter (10). Glutamate, on the other hand, is a signaling molecule involved in sustaining gut barrier

function and neuroendocrine reflexes. Glutamate serves as an important precursor for other bioactive molecules, including glutathione and proline (77). Taurine helps in structural and functional development of retina receptors and may also aid in fat absorption (78). Together, glutamine, glutamate, taurine and alanine make up 50% of the total free amino acids in HBM.

3.3.4.2 Creatine

Creatine is an essential metabolite needed for normal neural development (74). Neurological symptoms such as speech delay, intellectual disability and epilepsy are displayed in children with inborn errors of creatine synthesis or transport. Creatine helps to buffer cellular adenosine triphosphate (ATP) levels when energy demand is transiently greater than ATP synthesis rate (79). Creatine also serves as an energy vector between the sites of ATP synthesis and use. Creatine is spontaneously and irreversibly broken down to creatinine which is excreted in urine. Therefore, there is a continuous need to replace creatine to meet the growing infant's expanding tissue mass (79).

Infants receive their dietary creatine from mother's HBM. Edison et al (74), however, showed that a breast-fed infant only receives about 9% of the creatine needed from the diet (74). The remainder is provided through de novo synthesis in the infant in which creatine is produced from the amino acids, glycine and arginine.

3.4 Determinants of The Human Breast Milk Metabolome

Several maternal and infant factors have been identified as potential determinants of the HBM metabolome (Table 3.4). Some of these factors are explored further below.

1. **Mastitis:** Lactational mastitis is a lobular inflammation of the mammary gland caused by *Staphylococcus* spp. and/or *Corynebacterium* spp. with clinical symptoms including fever, chills and localized inflammation of the breast tissue (80). It often results in early cessation of EBF and may affect up to 33% of lactating women (68). Mastitis is associated with increased concentrations of the biogenic amines: spermine, putrescine, and histamine (68). Biogenic amines are usually produced due to the metabolic activity of certain microorganisms including some *Staphylococcus epidermidis* strains with activity for decarboxylation of amino acids; high concentration of biogenic amines in a mastitic breast could be due to increased decarboxylase activity (81). Mastitis is associated with an increased free fatty acid (FFA) concentration, although no change is observed in the total lipids and phospholipids, suggesting increased lipolysis within HBM (82). The release of FFA in an infected body site is one of the hallmarks of *Staphylococcus aureus* infection; elevated FFA may be a nonspecific immune response to bacteria-associated lactational mastitis (82, 83).

2. **Human Immunodeficiency Virus (HIV):** Normal HMO composition in HBM is disrupted in maternal HIV infection. Several studies show an increased abundance of 3'- sialyllactose (3′-SL) in HBM of HIV-positive mothers compared to HIV-negative mothers (50, 84, 85). Also, a higher proportion of 3′-SL amongst total HMOs was observed in HBM of HIV-negative women with lower CD4 counts (86). On the other hand, LNT and LNnT were more abundant in HBM of HIV-negative mothers (84).

   Among secretors, significantly higher absolute concentrations of 2'-FL, LNT and LNFP I and higher relative abundance of difucosyllacto-N-tetraose (DFLNT) and fucosyl-disialyllacto-N-hexaose (FDSLNH) was observed in HBM of HIV-infected women compared to uninfected secretor women (86). In non-secretors, only the relative abundance of DFLNH was higher in HBM of HIV-infected women compared with HIV-uninfected non-secretors (86).

   A higher proportion of 3′-SL per total HMOs in HBM has been associated with an increased risk of HIV transmission to infants and with markers of advanced HIV disease, while higher total HMO, LNnT and non-3'-SL HMO concentrations in HBM were associated with reduced vertical HIV transmission from mother to child (85). HMO composition of HBM has been found to influence the survival of HIV-exposed, uninfected (HEU) children born to HIV-infected mothers in Zambia up to 2 years of age (86). During the BF period, higher concentrations of 2′-FL, 3′-FL, and LNFP I, II/III were significantly associated with reduced mortality among HEU children (86).

3. **Maternal weight:** Compared to HBM of women with normal weight, HBM of overweight mothers had a higher abundance of saturated fatty acid, lower abundance of n-3 PUFA and also, a lower ratio of unsaturated to saturated fatty acids (87). Lower total polyamines have also been observed in HBM of obese mothers compared to HBM of mothers with normal body weight (71).

4. **Chemotherapy:** A case report has shown that DHA and inositol concentrations are reduced in HBM of a mother who underwent a chemotherapy cocktail for Hodgkin's lymphoma as compared with healthy controls (88).

5. **Maternal diet:** Dietary intake in the United States is skewed towards omega-6 fatty acids and associated with high content of LA in HBM of American mothers, and a particularly low DHA concentration (61). Unlike an omnivorous diet, a vegetarian diet provides high concentration of LA and ALA; hence a high concentration of these essential fatty acids in HBM of vegans (89).

6. **Maternal education:** In a study conducted by Nayak et al (2017) among low income families in an urban area in Bangladesh and India, higher level of maternal education was

associated with decreased saturated fatty acids, and increased ratio of polyunsaturated fatty acids to saturated fatty acids in HBM (90) possibly due to better informed food choices.

7. **Geographical location:** Significant differences in metabolite profiles have been observed based on geographical locations (91-94). With respect to HMOs, HBM of Chinese women had highest concentrations of 3′-FL and LNFP III, Spanish women had the highest concentration of 2'-FL, while the highest concentration of LNFP I was observed in HBM of women from Finland (91). Other metabolites including fatty acids, ethanolamine, n-6 PUFA, creatine, lactose and 2-oxoglutarate, pyruvate and lactate, methanol, polyamines, tyrosine have also been found to differ significantly between different geographical locations (91, 93, 94).

8. **Ethnicity:** Compared to Caucasian women, black women had higher HBM concentrations of 2-oxoglutarate, betaine and glycerophosphocholine and lower HBM valine concentration (93). HBM of Caucasians, on the other hand, had significantly lower concentrations of lactate and fucose compared to Asian women (93).

9. **Gestational age:** The HBM metabolite profile of full term babies shows higher concentrations of carnitine, caprylate, caprate, pantothenate, β-hydroxybutyrate and urea as compared with pre-term HBM, which has higher concentrations of lactose, phosphocholine, choline, glutamate, DHA, total polyamine, 3'-SL, and 6'-sialylactose (6'-SL) (69, 95, 96).

10. **Stage of lactation:** The composition and concentration of HMOs in HBM varies over the period of lactation (97, 98) with the mean total HMO concentration decreasing from 20.9 g/l in colostrum to 12.9 g/l in mature HBM at 4 months postpartum (99). The mean lactose concentration increases with period of lactation, from 56 g/L on day 4 to 68.9 g/L at 4 months postpartum (99).

11. **Course of lactation:** The last milk of a feed, the hind HBM, has been found to contain up to two or three times the total lipid concentration of foremilk (at the beginning of a feed) (23). Preterm low birthweight infants have been shown to exhibit increased weight gain when EBF hind HBM (100).

12. **Mode of delivery:** Though the impact of mode of delivery on metabolite profiles was found to be dependent on geographical location in a recent study (91), overall, women who had a vaginal delivery had significantly higher HBM concentrations of 3-hydroxybutyrate and LNFP III, while higher HBM concentrations of butyrate, urea, ethanolamine and proline were observed among women who underwent caesarean section (CS) delivery (91).

Table 3. 3: Factors Which May Influence the Human Breast Milk (HBM) Metabolome

Factors influencing human breast milk (HBM) composition	Metabolome	References
Maternal Health		
HIV	↑ 3′-SL, ↓ LNT ↓LNnT	(50, 84, 85)
Mastitis	↑ spermine, ↑ putrescine, ↑ histamine, ↑ FFA	(68)
Maternal weight		
Overweight mothers	↑ SFA, ↓ n-3 PUFA, ↓ Unsaturated FA: Saturated FA	(87)
Obese mothers	↓ Polyamines	(71)
Medication		
Chemotherapy	↓ Docosahexaenoic acid, ↓ Inositol	(88)
Sociodemographic factors		
Maternal diet		
Vegetarian diet	↑ LA, ↑ ALA, ↓ DHA	(61, 89)
Maternal education		
↑ Higher education	↑ PUFA: SFA	(90)
Geographical location		
Chinese women	↑ n-6 PUFA, ↓ SFA, ↑ 3′FL, ↑ LNFP III	(91, 92)
Spanish women	↑ 2'-FL, ↑ Putrescine	(91, 94)
Finland women	↑ LNFP I, ↑ spermidine	(91, 94)
South African women	↑ Lactose, ↑ 2-oxoglutarate, ↑ citrate	(93)
Infant factors		
Gestational age		
Pre-term	↑ DHA, ↑lactose, ↑ HMO conc., ↑phosphocholine, ↑choline, ↑glutamate, ↑3'-SL, ↑ 6'-SL, ↑ polyamine conc., ↓ spermidine/spermine	(69, 95, 96, 101-103)
Full term	↑carnitine, ↑caprylate, ↑caprate, ↑pantothenate, ↑β-hydroxybutyrate, ↑urea	(95)
HBM factors		
Lactational stage		

Colostrum	↑ HMOs, ↑ LNnT, ↑ 2'-FL, ↑ 3'-SL, ↓ Lactose, ↑ Leucine, ↑ Betaine, and ↑ Creatinine	(38, 95, 98)
Mature HBM	↓ Total HMOs, ↑ 3'-FL, ω6/ ω3 PUFA, ↑ oleic acid, ↑ palmitoleic acid, ↑ linoleic acid, ↑ tri-, di-, mono-glycerides, ↓ cholesterol, ↓ phospholipids, ↓ α-tocopherol, ↓ fucose, ↓ furanose isomers, ↓ D-glucosaminic acid, ↑ Alanine, ↑ caprylate, ↑ caprate, ↑ glutamate	(13, 21, 90, 95, 98, 104, 105)
Course of lactation		
Foremilk	↑ free amino acids, ↑ phenylalanine, ↑ threonine, ↑ valine, ↑ alanine, ↑ glutamine, ↑ serine	(100)
Delivery mode		
Vaginal delivery	↑ 3-hydroxybutyrate and ↑ LNFP III	(91)
CS delivery	↑ Butyrate, ↑ urea, ↑ ethanolamine and ↑ proline	(91)

↑: Increase concentrations, ↓ Decrease concentrations, SFA: Saturated fatty acids, PUFA: Polyunsaturated fatty acids; FFA: Free fatty acids; LNFP: Lacto-N-fucopentaose: 2'-FL: 2′-fucosyllactose; 3'-SL: 3'-sialyllactose; 6'-SL; 6'-sialyllactose; LA: Linoleic acid: ALA: α-Linolenic acid; DHA: Docosahexaenoic acid; LNT: lacto-N-tetraose LNT; LNnT: lacto-N-neotetraose; HIV: Human Immunodeficiency Virus.

3.5 CONCLUSION

HBM is a rich source of metabolites which contribute to HBM's beneficial properties, needed for optimal growth and development of infants. Our current knowledge of the HBM metabolome is largely restricted to studies from industrialized/westernized countries, with under-representation of samples from Sub-Saharan Africa. Further studies of geographically and ethnically diverse populations are needed.

A thorough understanding of HBM metabolites, their role in the developing infant and potential determinants is important for our understanding of its nutritional and bioactive value.

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CHAPTER 4

THE INFLUENCE OF DNA EXTRACTION AND MILK SKIMMING ON HUMAN BREAST MILK BACTERIAL PROFILES

Abstract

Culture-independent molecular techniques have advanced the characterization of environmental and human samples including the human breast milk (HBM) bacteriome. However, extraction of high-quality genomic DNA that is representative of the bacterial population in samples is crucial. Fat removal from HBM prior to DNA extraction is common practice but this may influence the bacterial population detected. The objective of this study was to compare four commercial DNA extraction kits and milk skimming in relation to HBM bacterial profiles. Four commercial DNA extraction kits, QIAamp® DNA Microbiome Kit, ZR Fungal/Bacterial DNA MiniPrep™, QIAsymphony DSP DNA Kit and ZymoBIOMICS™ DNA Miniprep Kit, were assessed using ten healthy donor breast milk samples. The kits were evaluated based on their ability to extract high quantities of pure DNA from HBM, and how well they extracted DNA from bacterial communities present in a commercial mock microbial community standard spiked into HBM. Finally, the kits were evaluated by assessing their extraction reproducibility. Bacterial profiles were assessed using Illumina MiSeq sequencing targeting the V4 region of the 16S rRNA gene. The ZR Fungal/Bacterial DNA MiniPrep™ and ZymoBIOMICS™ DNA Miniprep (Zymo Research Corp., Irvine, USA) kits extracted the highest DNA yields with the best purity. DNA extracted using ZR Fungal/Bacterial DNA MiniPrep™ best represented the bacteria in the mock community spiked into HBM. In un-spiked HBM samples, DNA extracted using the QIAsymphony DSP DNA kit showed statistically significant differences in taxa prevalence from DNA extracted using ZR Fungal/Bacterial DNA MiniPrep™ and ZymoBIOMICS™ DNA Miniprep kits. The only difference between skim and whole milk is observed in bacterial profiles with differing relative abundances of *Enhydrobacter* and *Acinetobacter*. DNA extraction, but not fat removal, substantially influences bacterial profiles of HBM samples, emphasizing the need for careful selection of a DNA extraction kit to improve DNA recovery from a range of bacterial taxa.

4.1 INTRODUCTION

There is a growing interest in the role that human breast milk (HBM) microbes play in infant and maternal health. The HBM microbiota has been shown to have a role in the development of the infant gut bacteriome and in promoting programming of the immune system (1, 2). Randomized clinical trials have shown that in mothers, these microbes could prevent and serve as an alternative treatment for lactational infectious mastitis caused by *Staphylococcus aureus* (3, 4).

Studies characterizing the bacterial diversity in HBM initially used bacterial culture, which has several limitations including detection of only viable organisms and being labour-intensive (5-7). Culture-independent molecular techniques, enabled by next-generation sequencing (NGS), can profile bacteria in complex environments and provide detailed phylogenetic information (8, 9). For this however, high quality genomic DNA that is representative of the microbial communities is required.

DNA extraction methods from samples such as faeces (10, 11), insects (12), soil (13), saliva (14) and colonic tissue (15) have been studied, yet, to our knowledge, no studies have documented optimization of DNA extraction from HBM samples. This is crucial as methodological variation such as the use of different DNA extraction kits may impact on microbial community profiling (16). Moreover, the effect of removing the fat layer of milk prior to DNA extraction is unknown, even though this approach is commonly used (17-19). Fat-rich tissue can cause difficulties in DNA extraction due to fat interfering with tissue disruption or by influencing the chemistry of the DNA isolation buffers (20).

Optimization of DNA extraction from HBM is necessary as HBM is known to have a relatively low bacterial biomass, and interfering substances (such as proteins) pose a challenge to the extraction of large amounts of quality DNA (21). In addition, the HBM bacteriome has been reported to contain a variety of gram-positive and gram-negative bacterial species (17) with differing cell wall composition which makes some species more difficult to lyse than the other. Improper lysis of these two groups of bacteria may result in a biased representation of the bacterial community present in HBM samples. Hence, methods need to be tested for their effectiveness, efficiency to lyse bacterial

cells, and the quality of the extracted DNA (12). We also incorporated a mock microbial community with predetermined DNA ratios from a mixture of bacterial species to assess bias of the DNA extraction kits.

The aim of this study was to compare and evaluate the extraction of bacterial genomic DNA from HBM samples using four commercial DNA extraction kits. Selection of kits used in this study was based on their availability and prior use for HBM bacteriome studies. Also, whole milk (WM) and skim milk (SM) were compared to determine whether the removal of the fat layer affected the bacterial population detected in HBM.

4.2 MATERIAL AND METHODS

4.2.1 Subjects and sample collection

HBM samples were collected from ten healthy lactating women residing in Cape Town, South Africa, after obtaining their consent. The women were asked to wash their hands, their nipples and surrounding breast area with soap and water. Milk was collected manually by hand expression into a 50 ml sterile collection bottle after discarding the first few drops. After collection, the samples were transported on ice and stored at $-20^{\circ}C$ until further processing. This study received ethical approval from the University of Cape Town Human Research Ethics Committee, South Africa (HREC REF: 649/2016).

4.2.2 Methods of DNA extraction

Each of the ten HBM samples was processed as un-spiked SM (n=10) and WM (n=10). DNA was extracted in duplicate from each SM and WM sample, using 4 different kits (total number of extracts=160, Figure 4.1A). In addition, as an extraction control, one HBM sample was divided into SM and WM, with each 1ml sample spiked with 75 µl (1x 10^9 cells) of Zymobiomics Microbial Community Standard ((ZMCS), (Catalogue no. D6300, Zymo Research Corp., Irvine, USA), Appendix 1, Table 1). Commercial ZMCS was employed as the mock community because it's readily available and affordable, and also manufactured by Zymo Research Corp., Irvine, USA, same company which manufactured two of the DNA kits assessed in the study. DNA was extracted

from replicas (on consecutive days) of the same spiked sample with each of the 4 kits (n=16) (Figure 4.1B).

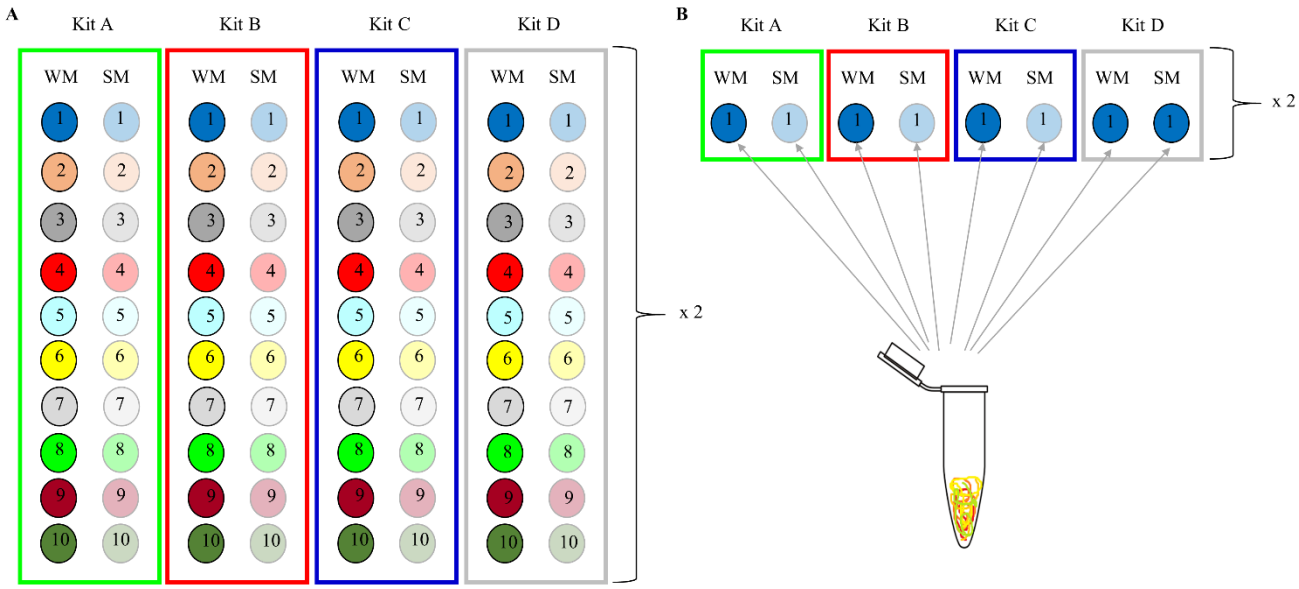

Figure 4. 1: Human breast milk samples collection per milk type and DNA extraction method. (A) DNA extraction from un-spiked skim milk and whole milk samples. (B) DNA extraction from spiked skim milk and whole milk samples.

HBM samples were homogenized by vortexing and SM was prepared according to a previously published protocol (22). In brief, the samples were centrifuged at 3500 g for 20 minutes at -10 °C, and the fat layer discarded. The supernatant was thereafter centrifuged at 7600 g for 10 minutes at room temperature, and the pellet used for further processing. The pellet from WM was prepared by centrifugation of the original milk sample at 7600 g for 10 minutes at room temperature. DNA was extracted from un-spiked HBM samples (n=160) and spiked HBM samples (n=16) using the recommended starting volume of the four different commercial DNA kits (Table 4.1).

Kit A: 500 µl Buffer AHL (host cell lysis buffer) was added to either 1ml of WM (or SM) for DNA extraction from WM and SM respectively.

Kit B: The pellet obtained from centrifugation of 1ml of WM (or SM), was resuspended in 250 µl of the resultant supernatant before proceeding to add 750 µl Lysis Solution (Zymo Research Corp., Irvine, USA) to the tube.

Kit C: The pellet obtained from centrifugation of 1ml of WM (or SM), was resuspended in 250 µl of the resultant supernatant before proceeding to add 750 µl Lysis Solution (Zymo Research Corp., Irvine, USA). An "off-board" mechanical lysis step followed using ZR BashingBead™ (Zymo Research Corp., Irvine, USA). Following mechanical lysis, the lysate was centrifuged at 5800 g for 1 min, and 400 µl of the supernatant was used for DNA extraction on the QIAsymphony® SP instrument (Qiagen, Hombrechtikon, Switzerland).

Kit D: The pellet obtained from centrifugation of 1ml of WM (or SM), was resuspended in 250 µl of the resultant supernatant before proceeding to add 750 µl ZymoBIOMICS™ Lysis Solution (Zymo Research Corp., Irvine, USA) to the tube.

An elution volume of 50 µl was used for all the kits except kit C in which the minimum elution volume was 60 µl as set by the supplier. For homogeneity and to ensure higher concentrations of DNA samples (as recommended by the manufacturer), 50 µl elution volume was used for Kit D. With the exception of Kit D which was eluted in DNase/RNase Free Water, DNA eluted from other kits were extracted in elution buffers. All bead-beating steps were performed in the TissueLyser LT (Qiagen) at a frequency of 50Hz for 5 min.

Table 4. 1: DNA extraction kits used in this study

Name of kit	Manufacturer's details	Extraction method	Kit name abbreviation	Recommended starting volume (µl)	Elution volume used (µl)
QIAamp® DNA Microbiome Kit	Qiagen, Hilden, Germany	Manual	kit A	1000	50
ZR Fungal/Bacterial DNA MiniPrep™	Zymo Research Corp., Irvine, USA	Manual	kit B	250	50
QIAsymphony DSP DNA Kit	Qiagen, Hilden, Germany	Automated	kit C	400	60
ZymoBIOMICS™ DNA Miniprep Kit	Zymo Research Corp., Irvine, USA	Manual	kit D	250	50

4.2.3 DNA quantification

The concentration and purity of DNA was measured using a NanoDrop™ ND-2000c Spectrophotometer (Thermo Scientific, Inc.). DNA yield was obtained by multiplying the DNA concentration by the final elution volume. The DNA yield from all samples was also assessed by 16S qPCR using a protocol previously described (23). Each 30 µl PCR reaction contained 2.5 µl DNA template, 1µl of probe, 15 µl Sensifast Probe No-rox (BIO-86020), 9.5 µl of MilliQ water and 1 µl each of 0.333 µM forward and reverse primer with conditions as described (Appendix 1, Table 2A). The qPCR was carried out on a 7500 Fast Real-Time PCR System (Applied Biosystems, CA 94404 USA). DNA was stored at -20 °C until further processing.

4.2.4 Extraction and sequencing controls

The respective elution buffer from each of the kits under investigation was processed as a negative extraction control. The ZMCS was extracted using all four kits and served as a positive extraction control. As per manufacturer's specifications, the genomic DNA abundance (%) for each bacteria species is 12% while the microbial OTU relative abundances are *Pseudomonas* spp. (4.6%), Enterobacteriaceae 1 (11.3%), Enterobacteriaceae 2 (10.0%), *Listeria* spp. (15.9%), *Staphylococcus* spp. (13.3%), and *Lactobacillus* spp. (18.8%) (Appendix 1, Table 1). DNA extraction on all HBM samples was done in duplicate on two consecutive days using the respective kits to evaluate extraction reproducibility. In addition, DNA extracts from two samples were randomly selected for repeat processing (library preparation and sequencing) to evaluate reproducibility of steps following DNA extraction.

In low biomass samples such as HBM samples, a portion of sequence reads may result from exogenous DNA contributed by reagent contaminants used during the process of DNA extraction and 16S rRNA gene library preparation. To allow *in silico* correction for contamination, cyanobacteria (*Arthrospira spirulina*) DNA extract obtained from a pure culture of Cyanobacterium (*Arthrospira spirulina*) , was spiked into DNA extracts from each of the respective negative controls (elution buffers) at a 16S rRNA gene concentration similar to that of HBM samples as assessed by qPCR. Since negative controls have little or no "competing" naturally present bacterial DNA, amplification of this small amount of background DNA may lead to over-estimation of the

contribution of contaminants to bacterial profiles. We compensated for this effect by spiking an amount of known bacterial DNA into control samples at an equivalent concentration to that found in HBM samples. These "cyanobacteria-spiked-elution buffers" were included in the library preparation and sequencing steps alongside the samples.

4.2.5 16S ribosomal ribonucleic acid (rRNA) amplicon library preparation

A two-step amplification approach described by Wu and colleagues (24) was employed to avoid PCR amplification biases associated with the use of adapter and index sequences. In the first PCR reaction, the hypervariable V4 region of the 16S rRNA gene was amplified using primers and PCR cycling conditions as previously described (25) (Appendix 1, Table 2B). Each 25.25 µl PCR reaction contained 12.5 µl 2X MyTaq™ HS Mix (BIO-25046), 2 µl of 0.8 µM forward and reverse primers, 1 µl of MilliQ water, 0.75 µl dimethyl sulphoxide (catalogue no D4540, Sigma-Aldrich®, MO, USA) and 7 µl bacterial genomic DNA.

In the second PCR reaction, the same reagents were used as above, except that the template was 7 µl of the amplicon product from the first PCR reaction, and the reverse primers contained Illumina adapters and various unique index sequences at the 3' end for each sample (26). The PCR conditions are the same with the short PCR run as described except for an additional 20 cycles in the PCR run (25) (Appendix 1, Table 2C).

Amplicon products were cleaned with Agencourt SPRIPlate 96 super Magnet Plate and QuantiFluor™ dsDNA System was used to quantify cleaned amplicons (25). The integrity of the cleaned amplicons was checked by UV illumination of the gel. Briefly, 5 µl of each cleaned amplicon was analyzed on a 2% agarose gel containing 1% ethidium bromide. Amplicons were normalized by pooling at an equimolar concentration of 100 ng, and purified using Agencourt AMPure system (Beckman Coulter, UK). Pooled library was extracted on 1.5% agarose gel. QIAquick Gel Extraction Kit (Qiagen, MA, USA) was used for gel purification with the following minor modification to manufacturer's protocol. The elution buffer, Tris-EDTA buffer (pH 8.0), was heated at 70 °C to improve amplicon recovery (step 13). Qubit® dsDNA BR Assay Kit was used for final quantification of the pooled 16S library.

4.2.6 16S ribosomal ribonucleic acid (rRNA) gene sequencing

The pooled 16S library was paired-end sequenced on the Illumina MiSeq® system using the MiSeq® Reagent v3 kit (600 cycles). The quality control steps entailed 1) the quantitation of adapter-ligated dsDNA and 2) analysis of fragment size of the pooled library using the KAPA Library Quantification Kits (Illumina®) (KAPA Biosystems, MA, USA) and Agilent High-Sensitivity (HS) DNA Kit (Agilent Technologies, CA, USA) respectively. The library pool was thereafter diluted to 4nM and denatured using Buffer EB (Qiagen) and 0.2 N NaOH respectively. The mixture was neutralized and further diluted with HT1 Buffer (Illumina®) to 5.5 pM which was loaded to the sequencer according to the manufacturer's instructions (27), alongside the sequencing control (PhiX library) spiked into the 16S library at 15% (v/v).

4.2.7 Bioinformatics workflow

The sequencing quality of FASTQ files was assessed using FASTQC (v0.10.1) package (28). Forward and reverse sequences were then merged using UPARSE (v7.0.1090) allowing 3 mismatches in overlaps (fastq_maxdiff set to 3), followed by quality filtering using USEARCH9 fastq_filter (sequences truncated to 250bp). Reads with a maximum expected number of error >0.1 were discarded (fastq_maxee set to 0.1) (29). De-replication and selection of sequences occurring more than once was performed by sortbysize command in USEARCH9. Clustering of sequences into operational taxonomic units (OTUs) (with a clustering radius of 3) was done using USEARCH9 cluster_otus command. The USEARCH9_uchime2_ref tool was used to detect and remove chimeras and OTU counts were obtained using USEARCH9 usearch-global (30).

Decontamination of biological samples was done by first removing cyanobacteria sequences from the four "cyanobacteria-spiked-NTC" controls. Sequences remaining after the removal of cyanobacteria sequences were identified as "contaminant sequences". The latter were screened against biological samples by aligning biological sample sequences to spiked control sequences at 100% similarity using align_seq.py, based on PyNAST (31). An average number of reads was calculated for each of the "contaminant sequences" matching at 100% similarity to biological sample sequences. "Contaminant sequences" were removed from biological samples by removing the average number of reads calculated from the four "cyanobacteria-spiked-NTC" controls.

Further processing of data was performed using Quantitative Insights Into Microbial Ecology (QIIME) 1.9.1 suite of software tools (32). OTU picking was occurred at 97% sequence similarity and taxonomic assignment was carried out against SILVA database (Version 132.) (33) using RDP classifier (v2.2) in QIIME (v1.9.1) (32). Rarefaction plot of Shannon diversity against sequencing depth was also generated in QIIME using alpha_rarefaction.py (32). The raw sequencing reads were deposited in the NCBI Sequence Read Archive (SRA) database with accession number PRJNA510564.

4.2.8 Data analysis

Data analysis and graphical illustrations of the data (bar plots, boxplots, dendograms) were generated in R statistical package (version 3.4.1) and R studio 1.1.456 (34). Agglomerative cluster dendograms were generated by complete linkage hierarchical clustering (35) using the [hclust] function (36). This hierarchical clustering method is based on the Bray-Curtis dissimilarity index (37) of the R *vegan* package (38). Cluster dendogram was performed for all OTUs with relative abundance of > 0.5%. Alpha diversity within each sample was measured using the Shannon–Weaver index with function [diversity] in the R package *vegan* (39), which measures both the richness and evenness of organisms within a given sample. Analysis of Variance Table (Type II tests) (40) was used to test the significance difference in alpha diversity between groups and to generate a p-value with a significance threshold of $p < 0.05$, while error estimates were based on Pearson residuals. Log-ratio biplots using a Bayesian prior technique for adjustments of zero counts were made as previously described (41) and employed lambda-scaling to ensure evenness in the "total spread" of the data sets (42). Log-ratio biplots were used to show multivariate clustering patterns as they are specific for proportions/percentages (43).

Generalized linear models (GLM) were used to test the effect of SM and WM, and the four DNA extraction kits on HBM bacterial profiles at different taxonomy levels. The negative binomial distribution (44) in the package stats with the Quasi-Poisson family function (45) was applied to model over-dispersion. "Breast milk" bacteria profiles were removed from the HBM sample spiked with ZMCS to investigate the eight bacteria genera expected in the ZMCS. Following this, bacteria genera in the known "theoretical" ZMCS were compared with similar bacteria profiles in the HBM

sample spiked with ZMCS. Benjamini–Hochberg method for multiple correction was used to correct all *p*-values, set at a 5% significance level, by the false discovery rate (FDR) (46). Tukey's Honest Significant Differences (HSD) method was used to generate a single-step multiple comparison of means procedure with 95% family-wise confidence intervals (47). Notched box-plots (48) were made to show distribution analysis of the data, as they display the 95% confidence interval for the median.

4.3 RESULTS

4.3.1 Influence of DNA extraction kits and milk skimming on yield and quality of DNA extracted from un-spiked human breast milk samples

The efficiency of four DNA extraction kits were compared based on yield and purity of the extracted DNA from un-spiked HBM samples (n=160) with NanoDrop™ ND-2000c Spectrophotometer (Figure 4.2). A significant difference in the yield of DNA extracted was observed between the kits (p = 8.71e^{-09}) (Figure 4.2A). Kits B and D gave the highest DNA yield; Tukey's HSD revealed no significant difference between these two kits (p = 0.96). No significant difference was observed in DNA yield when comparing SM and WM (Appendix 1, Figure 1A). However, when comparing bacterial 16S DNA concentration from HBM samples, using qPCR, no significant difference was observed between kits (p=0.253) (Appendix 1, Figure 2A). Similarly, no significant difference was observed in 16S DNA concentration when comparing SM and WM (p=0.524) (Appendix 1, Figure 2B).

DNA purity was assessed using the 260/280 absorbance ratio measure and no significant differences were observed between the four kits (p = 0.327) (Figure 4.2B) though the purity of DNA varied between the kits. Kits B and D had DNA purity closest to the recommended range of 1.8-2.0, while kit C showed a large variation in DNA purity between samples. There was no significant difference in DNA purity between SM and WM (Appendix 1, Figure 1B).

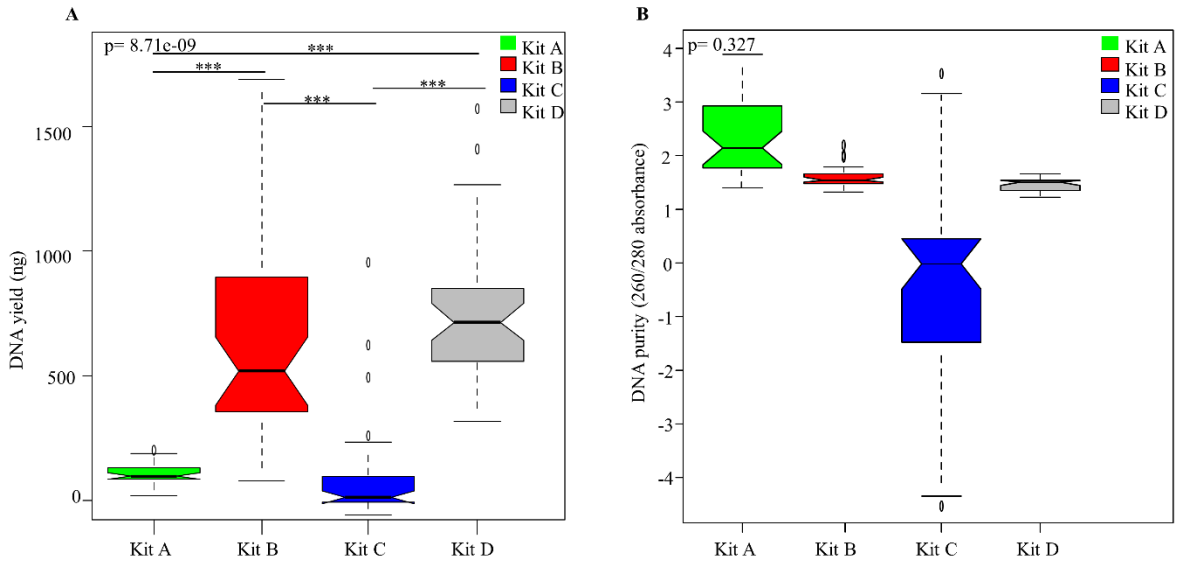

Figure 4. 2: DNA yield and purity of the four different commercial kits. Notched box plots showing (A) the DNA yield and (B) the DNA purity (260/280 absorbance) obtained by using each of the four kits. The notched box signifies the 75% (upper) and 25% (lower) quartile showing the distribution of 50% of the samples. The line inside the box plot represents the median, and the notch the 95% confidence interval for the median. The whiskers (top and bottom) represent the maximum and minimum values. Outliers, which are beyond 1.5 times the interquartile range above the maximum value and below the minimum value, are shown with open circles. *** represents $p<0.001$.

4.3.2 Influence of DNA extraction kits and milk skimming on bacterial profiles obtained from mock microbial community standard spiked into human breast milk

To evaluate which DNA extraction kit best extracted the bacterial communities in the known ZMCS community, composition and abundance were assessed after spiking this community into WM and SM of sample 1 (extracted by each of the 4 kits in duplicate) (Appendix 1, Figure 3B). When comparing the bacterial 16S DNA concentration in the spiked vs. un-spiked sample, >99.7% of the total 16S DNA within the spiked sample originated from the ZMCS (Appendix 1, Table 3B), and therefore, the contribution of the indigenous bacterial flora of this sample to the profiles generated from this sample would be negligible.

Hierarchical cluster analysis was used to create a dendogram of the bacterial composition of the ZMCS spiked into the HBM sample alongside the community profile provided by the manufacturer (Appendix 1, Figure 3B). Bacterial profiles did not cluster based on whether DNA was extracted from WM or SM, but rather based on the DNA extraction kit used. Overall, kit A showed a very different profile compared to ZMCS and the other three kits under investigation. Kit A only

extracted DNA from three of the eight bacterial genera/families present in ZMCS. The three other kits (kits B, C and D) represented all the eight bacterial genera/families albeit in differing abundance. Kit C showed the widest variation in composition between replicates, with the samples clustering on different clades of the dendogram. Kit B (and some replicates for kit C) clustered closest to the ZMCS suggesting the best representation of the microbial community standard.

Beta diversity (Bray-Curtis dissimilarity index) was also computed to show the differences in bacterial composition between the composition of ZMCS and DNA extracted from each kit for WM and SM spiked samples. A lower beta diversity value would mean greater similarity between the composition of ZMCS and the HBM sample spiked with ZMCS. For SM, kit B had the lowest beta diversity of 0.06, meaning it most closely represented the bacterial profiles of the ZMCS. For WM, the lowest beta diversity of 0.16 was seen for kits B and C (Appendix 1, Table 4).

We further evaluated the differences in relative abundances of the eight bacterial genera/families expected from the known "theoretical" bacterial profile of ZMCS and those resulting from the extraction kits (Figure 4.3). Gram-negative organisms present in ZMCS were substantially under-represented in samples extracted with Kit A (Figure 4.3 A-C). Kit B best represented the gram-negative organisms in the known mock community with relative abundances closest to the mock community (Figure 4.3 A-C). The relative abundances of gram-negative organisms in the ZMCS was over-represented in DNA extracted using Kit C and Kit D (Figure 4.3 A-C). In relation to the five gram-positive organisms, no kit showed an ideal representation of all (Figure 4.3 D-H). Kit B and Kit C resulted in the closest proportional representation of *Lactobacillus* spp. to the ZMCS. On the other hand, they showed a lower relative abundance of *Listeria* spp. and *Staphylococcus* spp. compared with ZMCS. There were no differences in relative abundances of taxa in ZMCS extracted from SM vs. WM (Figure 4.4 A-H).

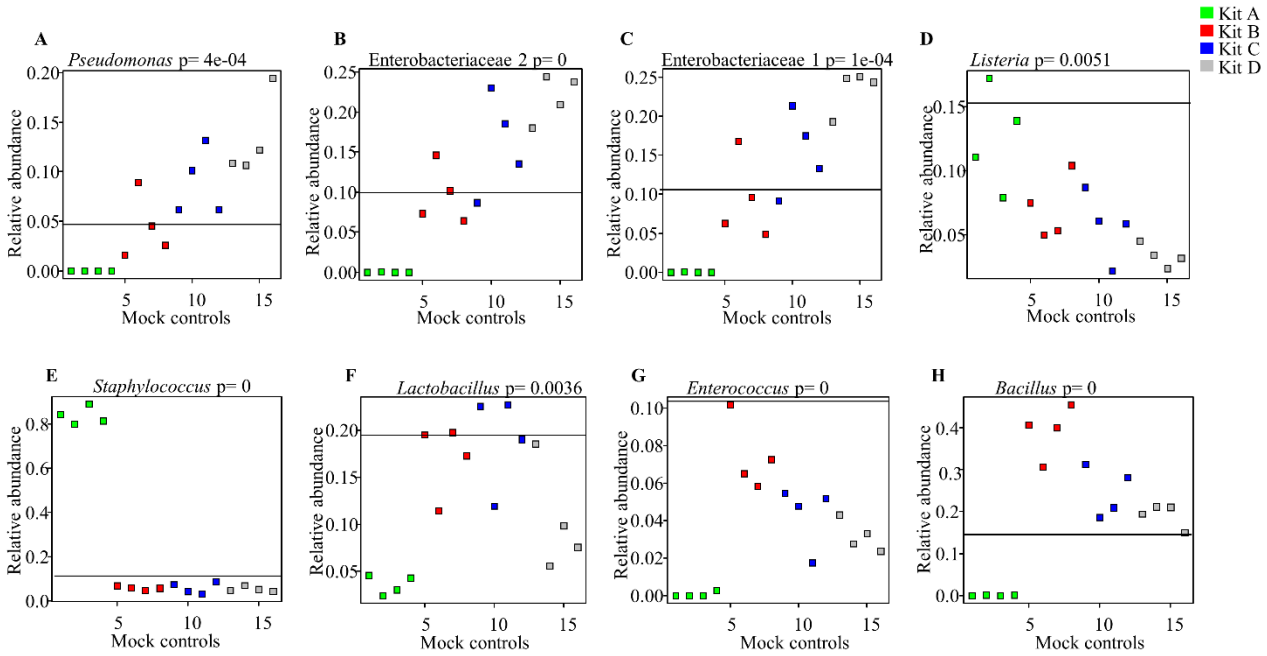

Figure 4. 3: Relative abundances of bacterial taxa extracted by kits in comparison to the commercial ZMCS composition. The horizontal line in each box indicates the relative abundance of the organism in question in the commercial ZMCS as given by the manufacturer. P values refer to differences in relative abundance of each bacterial taxon between different kits ($p < 0.05$: *; $p < 0.01$: **). P-values were generated by Anova test in stats package of R and adjusted using Benjamini–Hochberg's false discovery rate. ZMCS= Zymobiomics Microbial Community Standard.

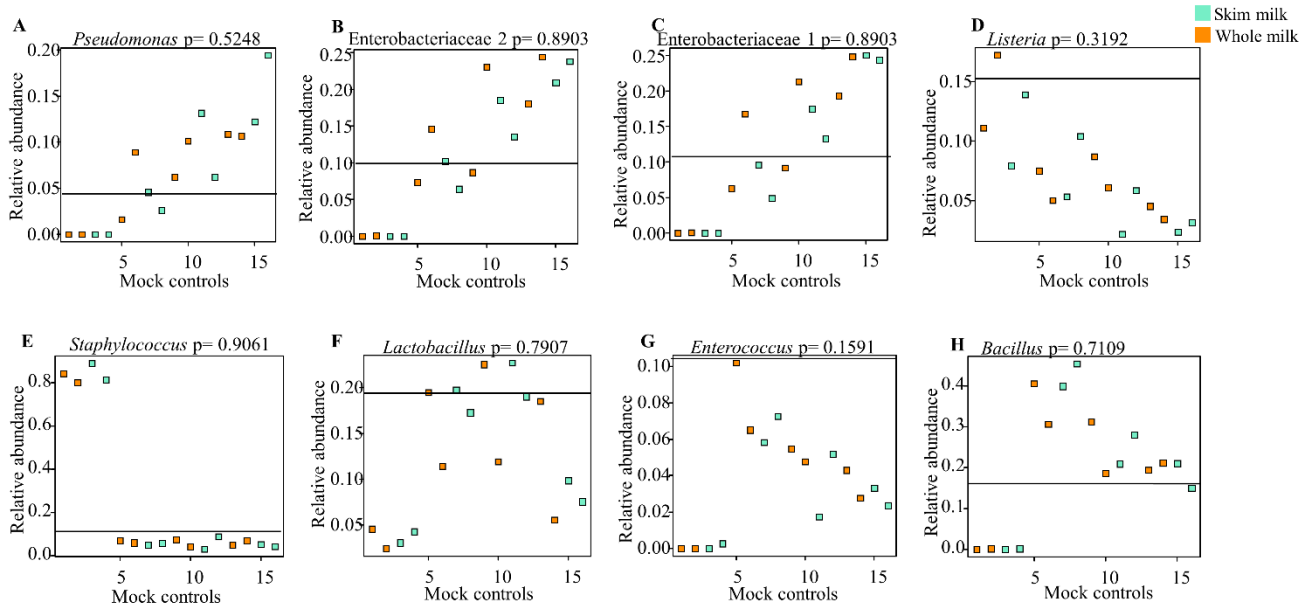

Figure 4. 4: Relative abundances of bacterial taxa extracted by skim milk and whole milk in comparison to the commercial ZMCS composition. The horizontal line in each box indicates the relative abundance of the organism in question in the commercial ZMCS as given by the manufacturer. P > 0.05 shows a non-statistically significant difference in relative abundance of each bacterial taxon in the HBM sample spiked with ZMCS as extracted by skim milk and whole milk. P-values were generated by Anova test in stats package of R and adjusted using Benjamini–Hochberg's false discovery rate. ZMCS= Zymobiomics Microbial Community Standard.

Due to the poor performance of kit A in extracting DNA from *Pseudomonas* spp., Enterobacteriaceae, *Enterococcus* spp. and *Bacillus* spp. in the HBM sample spiked with ZMCS, the spiked sample were re-extracted with kit A, however on this occasion, the first steps of the manufacturer's guidelines involving the initial host DNA depletion step (benzonase treatment) were omitted; a decision based on communication with the manufacturer. The benzonase steps are intended to deplete extracellular bacterial DNA. This modification of the extraction protocol resulted in substantially improved representation of the bacteria in the ZMCS (Figure 4.5). However, at this point, kit A was excluded from further analysis of (un-spiked) HBM samples.

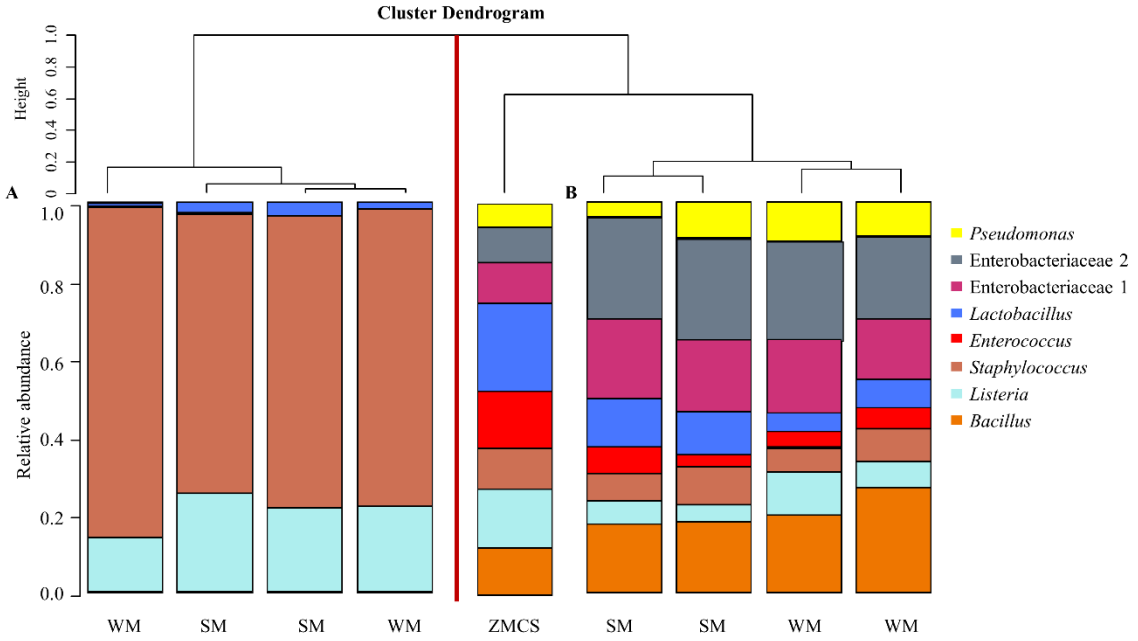

Figure 4. 5: Complete linkage hierarchical clustering showing the relative abundances of bacterial taxa in the HBM sample spiked with ZMCS. (A) Kit A DNA extraction protocol as per manufacturer's recommendations and (B) Kit A DNA extraction protocol in which the initial steps (steps 1 to 4) involving degradation and digestion of host DNA were omitted. Samples were processed in duplicate. WM-whole milk, SM-skim milk. ZMCS: Zymobiomics Microbial Community Standard reference.

4.3.3 Influence of DNA extraction kit and milk type on bacterial diversity in un-spiked HBM samples

A total of 3,559,506 high-quality filtered raw paired-end reads was obtained from 160 HBM samples. The number of post-filtered reads after the removal of contaminant sequences was 263,464. The median and mean sequence read count per sample was 1,405 and 1,670 (range 163-5,681) respectively**.** Alpha rarefaction curves showed that Shannon diversity plateaued at a sequence depth of 100 (Appendix 1, Figure 4).

The dominant phyla in HBM samples were Firmicutes and Proteobacteria, followed by a relatively low abundance of Actinobacteria and Cyanobacteria. At the genus level, the predominant bacteria were *Staphylococcus* spp., *Streptococcus* spp., *Lactobacillus* spp., *Acinetobacter* spp., and members of the family Enterobacteriaceae (Appendix 1, Figure 5).

The influence of DNA extraction kits and extraction from SM vs. WM were compared with respect to bacterial alpha diversity (Shannon diversity index). Overall; there were no significant differences

in alpha diversity between DNA extracted by different kits (p=0.11) (Figure 4.6). Tukey HSD multiple comparisons of means also showed no significant difference in alpha diversity of samples between each of the kits. Similarly, there were no statistically significant differences in alpha diversity of DNA extracted from WM vs. SM (p=0.88) (Figure 4.6). Therefore, extraction kit and milk type did not influence the alpha diversity of bacterial composition in HBM samples.

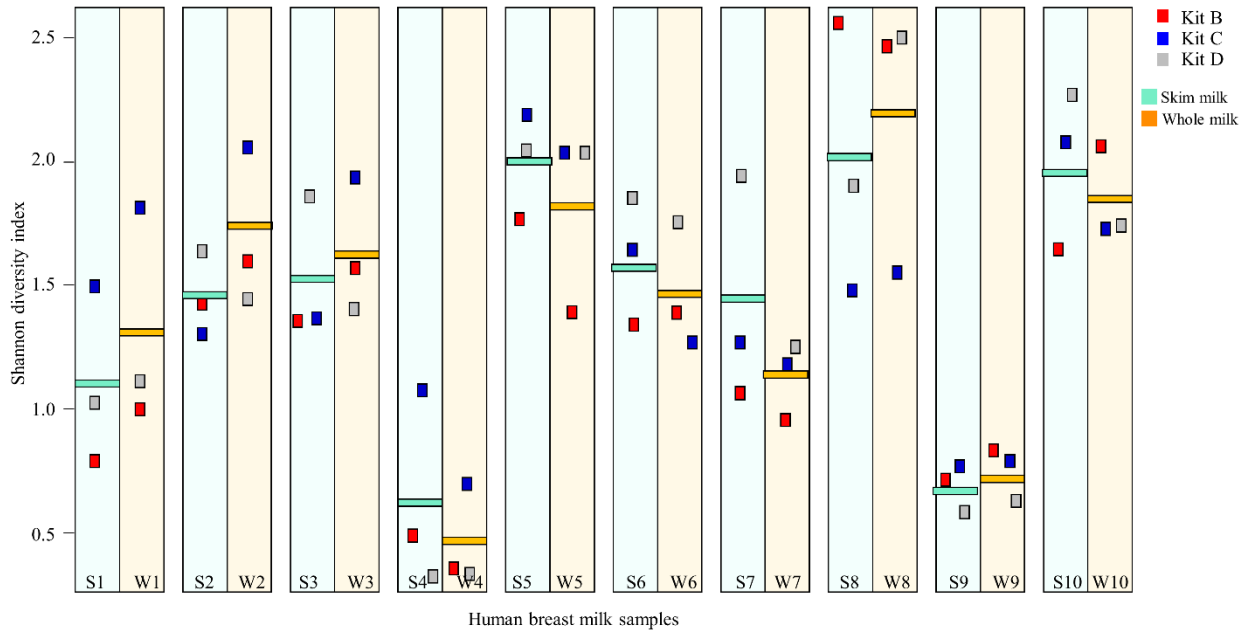

Figure 4. 6: Shannon diversity index of the un-spiked HBM samples based on kits and milk type. Each HBM sample is represented by a rectangle. The horizontal cyan and orange lines represent bacterial alpha diversity from skim milk and whole milk respectively. The alpha diversity in bacterial DNA extracted by each kit is indicated by coloured boxes. Kits B, C and D are represented by red, blue and grey lines respectively. WM=whole milk, SM=skim milk.

Unsupervised hierarchical clustering of samples based on Bray-Curtis dissimilarity index showed clustering largely based on HBM donor, rather than on the basis of DNA extraction kit used (Figure 4.7).

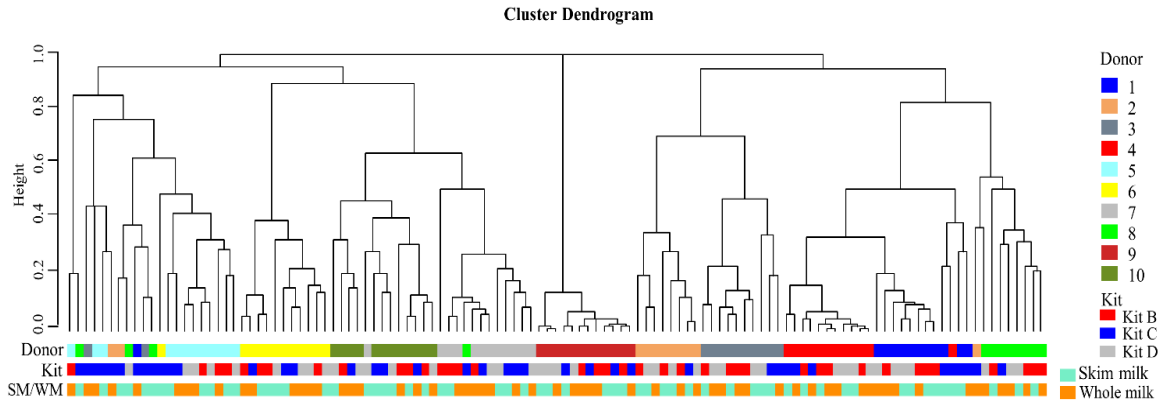

Figure 4. 7: HBM samples cluster primarily based on the donor. Complete linkage unsupervised hierarchical clustering based on the relative abundances of bacteria genera in un-spiked HBM samples show that DNA extraction kits and milk type affects bacterial profiles less when compared to the source. W: whole milk, S: skim milk; 1-10 represents each donor; kit B: ZR Fungal/Bacterial DNA MiniPrep™, kit C: QIAsymphony DSP DNA Kit, and kit D: ZymoBIOMICS™ DNA Miniprep Kit.

Furthermore, exploration of bacteria with abundances > 0.5% using log ratio biplots did not show clustering of HBM samples associated with either DNA extraction kit used (B, C or D) or milk type (SM or WM) either at the phylum level (Appendix 1, Figure 6A-B) or genus level (Appendix 1, Figure 7A-B), suggesting that neither extraction kit nor use of WM vs. SM were major drivers of differences between samples. In contrast, clustering was observed among samples from each donor, irrespective of the kit used for extraction or whether WM or SM was extracted (Appendix 1, Figure 6C; Appendix 1, Figure 7C). Therefore, donor, rather than extraction kit or milk type, was the major determinant of bacterial profiles identified in HBM samples.

GLM and Turkey HSD were used to compare the different taxa observed in HBM samples in relation to DNA extraction kit used and between WM and SM. Appendix 1, Table 5 summarizes the mean relative abundance of statistically significant bacteria at the different taxonomy levels for the kits, and WM and SM. Comparison of different bacteria taxa between samples processed with the different DNA extraction kits showed significant differences in relative abundances of six bacterial genera (*Ralstonia* spp., *Pseudomonas* spp., *Haemophilus* spp., *Acinetobacter* spp., OTU_52 belonging to the family Rhodobacterales, and *Lactobacillus* spp.), while WM and SM differed only for two bacterial genera, *Enhydrobacter* spp., and *Acinetobacter* spp., both belonging to the family Moraxellaceae. No significant differences in relative abundances were observed between Kit B and Kit D at any taxonomy level.

4.3.4 Reproducibility of extractions

DNA extraction was carried out in duplicate, on consecutive days to evaluate the reproducibility of each DNA extraction kit. The total read count of each OTU in each duplicate set (per kit and per milk type) was tested using multiple R-squared, representing the proportion of variance reproduced by replicating the reads.

Reproducibility (R^2) ranged from 0.4751 to 0.9483 (Appendix 1, Table 6). For WM, kit D produced the most reproducible results with R^2 of 0.9483, followed by kit C with R^2 of 0.7421 while kit B produced the least reproducible results with R^2 of 0.4908. For SM, kit D produced the most reproducible results with R^2 of 0.7976, followed by kit B with R^2 of 0.7581 while kit C produced the least reproducible results with R^2 of 0.4751. We observed wide variations in reproducibility of SM and WM for kit B and kit C, unlike kit D where reproducibility was better, independent of milk type. The multiple R^2 generated for the two sequencing controls included during the 16S library preparation was 0.9713.

4.4 DISCUSSION

We compared different methods of bacterial nucleic acid extraction from HBM prior to 16S microbiome profiling, including the effect of removing the fat layer. To our knowledge there are no published studies exploring this method. We observed a significant difference in DNA yield and purity, relative abundance of specific taxa, and the ability to represent the bacteria composition in a known mock microbial community when different DNA extraction kits were employed to extract DNA from HBM samples. On the other hand, skimming of milk prior to DNA extraction had limited impact on bacterial profiles, only showing a small influence on the relative abundance of two specific taxa within the phylum Proteobacteria.

In agreement with previously published literature (2, 17, 49), HBM has a vast population of bacterial communities in healthy individuals and dominated at the phylum level by Firmicutes and Proteobacteria, followed by Actinobacteria. At the genus level, we found that *Staphylococcus* spp., *Streptococcus* spp., *Lactobacillus* spp., *Acinetobacter* spp., and members of the family Enterobacteria were the dominant members.

DNA extraction has been validated for several bacteriome studies including feces, oral cavity, turkey cecum and soil using evaluation criteria such as DNA yield and purity, DNA shearing, reproducibility, quantitative PCR (5, 15, 50-52), 16S rRNA gene sequence-based taxonomic signatures (50-52), bacterial community fingerprints (10, 16), and custom designed phylogenetic microarray profiles (15). However, to our knowledge, no study has previously compared different DNA extraction methods for HBM.

In this study, DNA extraction methods were evaluated based on DNA yield and purity, bacterial diversity and reproducibility. HBM has a high fat content which may present challenges in extraction of DNA for molecular testing (21, 53). Furthermore, given the low microbial biomass present in HBM, it would be expected that the bacterial DNA yields would be substantially lower than those of other specimen types, for example faecal specimens, posing a challenge for 16S rRNA based studies (54). Despite these potential complications, high DNA yields and within-range DNA purity were achieved by kits B and D, both from Zymo Research Corp., Irvine, USA.

Low biomass specimens are prone to contaminating microbial DNA which is ubiquitous within DNA extraction kits, PCR reagents and other laboratory reagents. (55, 56). This contaminating DNA may distort the relative abundances of microbial composition in datasets, thereby leading to erroneous interpretations (55). We therefore mitigated this effect by in-silico decontamination of our biological samples. The variation experienced with kit C might be because the DNA yields were very low, thus a higher risk of cross-contamination.

In addition to the above criteria, we also evaluated the kits based on representation of the bacterial profile in a commercial mock microbial community standard. A bacterial community with a defined composition and abundance (5) is unlike environmental or human samples, which can be complicated by an unknown type and number of indigenous microorganisms. In the study by Willner and colleagues, a mock community was also used in identifying methodological contaminants, identifying detection limits and empirical cut-off values for 16S pyrosequencing in order to filter out spurious OTUs, and also to compare method reproducibility (57).

Using 16S rRNA gene sequencing we compared the ability of four commercial DNA extraction kits to recover DNA from bacteria from a known commercial mock microbial community containing

eight bacterial species, which had been spiked into HBM by comparing the expected bacterial profiles in the HBM sample spiked with ZMCS to the theoretical bacterial composition of the mock microbial community standard. Importantly, the proportion of the total 16S bacterial DNA within the spiked samples was derived almost exclusively (>99.7%) from the mock community, and so one would not expect these profiles to be affected by the indigenous bacterial community within the spiked HBM sample. Whilst extraction using three of the kits resulted in reasonable representation of the mock community composition, as with other studies (5, 51), significant differences in bacterial profiles were shown when DNA from mock bacterial communities was extracted with different kits.

Gram-positive organisms are more difficult to lyse; however, kits B, C and D showed an over-representation of the genus Bacillus despite an under-representation of similar hard-to-lyse bacteria like Enterococcus. In a study by Yuan et al. (2012) which evaluated six commonly used DNA extraction procedures from the Human Microbiome study, the observed relative abundance of *Lactobacillus iners* in a mock community was higher than its expected relative abundance (5). In a previous study which evaluated the influence of four DNA extraction methods on oral bacterial profiles, only one method was able to represent all bacteria in the mock community (51). Overall, kits B, C and D represented all eight expected bacterial genera/families, though in differing abundance.

Kit A performed markedly differently from the other kits. This kit had a considerably lower DNA yield, perhaps because the initial steps incorporate the depletion of human DNA to yield enriched bacterial DNA. This supports the report of Wen et al. (2016) in which a microbial DNA extraction method with pre-treatment of depletion of host nucleic acid by benzonase resulted in significantly lower DNA concentrations of samples (58). Bacterial profiles in HBM spiked with the mock community and extracted with Kit A revealed a biased composition with over-representation of gram-positive organisms, with *Staphylococcus* spp. dominating. This may have been due to lysis of gram-negative bacteria during the early extraction steps, and subsequent depletion by benzonase treatment. On repeating this extraction, but omitting the initial benzonase treatment steps, a good representation of all the eight bacterial genera in the mock standard was shown. It's therefore

imperative that researchers fully understand the importance of each step in any DNA extraction protocol and its impact on the bacterial community of their samples.

We showed no impact of DNA extraction kit on alpha diversity (reported using the Shannon-diversity index), as has previously been shown on salivary bacterial communities (14). When considering beta diversity (reported using Bray-Curtis dissimilarity index), samples clustered together based on donor, irrespective of the DNA extraction kit used or whether HBM was skimmed or not prior to DNA extraction. A study evaluating variation in human gut microbiota profiles due to DNA extraction methods, confirmed this. In this study, the main source of variation in a dissimilarity matrix was related to donor, followed by the DNA extraction method (16). Another study by Wesolowska-Andersen et al. (2014) which assessed the effect of bacterial DNA extraction method from faeces, observed a higher inter-individual variation than that seen for the extraction method (59).

We observed in our study that DNA extraction kits had an impact, albeit relatively minor, on the relative abundances of specific bacterial taxa from extracted DNA of samples. Other studies have also observed significant differences in relative abundances of bacterial taxa based on DNA extraction methods for faecal specimens (59, 60).

Though a major strength of this study is the use of mock microbial community, this study was faced with some limitations. One is that the bacterial composition in the commercial mock community is not a true reflection of microbes usually found in HBM. However, seven bacterial genera that are commonly isolated in HBM were represented in the mock standard at the genus level. A further limitation is associated with the use of 16S rRNA gene targeted sequencing which gives low phylogenetic resolution power of bacteria up to the species level. Also, the mock microbial community was not extracted independently and processed by itself. Lastly, the “theoretical” bacterial profiles may differ from our experimental profiles due to factors such as the use of different 16S gene variable region and sequencing approaches.

4.5 CONCLUSION

In summary, we have shown that DNA extraction method, but not fat removal, has an important influence on characterization of the HBM bacteriome by 16S rRNA sequencing. Detailed understanding of the impact of individual steps within an extraction procedure is required to prevent significant bias in determining the composition of the HBM bacteriome.

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CHAPTER 5

INFLUENCE OF SOCIO-ECONOMIC AND PSYCHOSOCIAL PROFILES ON THE HUMAN BREAST MILK BACTERIOME OF SOUTH AFRICAN WOMEN

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Author Contributions

Conceptualization, M.P.N., D.J.S., and H.J.Z.; methodology, S.C.-W.; software, K.S.M.; formal analysis, S.G.-L. and A.O.-O.; investigation, A.O.-O.; resources, M.P.N. and H.J.Z.; writing—original draft preparation, A.O.-O.; writing—review and editing, S.C.-W., K.S.M., S.G.-L., D.J.S., M.P.N., H.J.Z., and E.d.T.; visualization, A.O.-O.; supervision, M.P.N. and E.d.T.; funding acquisition, M.P.N. and H.J.Z.

Abstract

The human breast milk (HBM) bacteriome is an important, continuous source of microbes to the neonate in early life, playing an important role in shaping the infant's intestinal bacteriome. Study of the composition of the HBM bacteriome is an emerging area of research, with little information available, particularly from low- and middle-income countries. The aim of this study was to characterize the diversity of bacterial communities in HBM samples collected between 6-10 weeks postpartum from lactating South African women, and to study potential influencing factors of the bacteriome. Using 16S rRNA gene sequencing of samples from 554 women, we demonstrated that the HBM bacteriome was largely dominated by the phyla Firmicutes (mean relative abundance: 71.1%) and Actinobacteria (mean relative abundance: 16.4%). The most abundant genera identified from the HBM bacteriome were *Streptococcus* (mean relative abundance: 48.6%), *Staphylococcus* (mean relative abundance: 17.8%), *Rothia* (mean relative abundance: 5.8%) and *Corynebacterium* (mean relative abundance: 4.3%). "Core" bacterial genera including *Corynebacterium*, *Streptococcus*, *Staphylococcus*, *Rothia*, *Veillonella*, *Gemella*, *Acinetobacter*, *Micrococcus* and a genus belonging to the family, Enterobacteriaceae, were present in 80% of samples. HBM samples were classified, according to their bacteriome, into three major clusters, dominated by the genera *Staphylococcus* (cluster 1), a combination of *Staphylococcus* and *Streptococcus* (cluster 2) and *Streptococcus* (cluster 3). The cluster groups differed significantly for Shannon and chao1 richness indices. Bacterial interactions were studied using co-occurrence networks with positive associations observed between the abundances of *Staphylococcus* and *Corynebacteria* (members of the skin microbiota) and between *Streptococcus*, *Rothia*, *Veillonella* and *Gemella* (members of the oral microbiota). HBM from older mothers had a higher Shannon diversity index. Study site was associated with differences in HBM bacteriome composition (PERMANOVA, $p < 0.05$). No other tested socio-demographic or psychosocial factors were associated with HBM bacterial composition.

Keywords: Human breast milk; bacteriome; microbiome; 16S rRNA gene sequencing; bacterial profiles; socio-economic; psychosocial; Africa.

5.1 INTRODUCTION

The bacterial community (the bacteriome) present in human breast milk (HBM) is diverse and plays an important role in the health of both mothers and their infants (1, 2). HBM bacteria are one of the main sources of continuous microbes to the infant in early life and play a role in shaping the infant's intestinal bacteriome (3). Several bacterial genera, including *Streptococcus, Staphylococcus, Corynebacterium, Pseudomonas,* and lactic acid bacteria (LAB; *Lactobacillus* spp. and *Bifidobacterium* spp.) have been identified as key components of the HBM bacteriome, with the phyla Firmicutes and Proteobacteria typically dominating (4-6).

The concept of a core bacteriome has been proposed (7), with *Staphylococcus* and *Streptococcus* uniformly present (1, 8, 9), but with variation in other genera (10). Despite a core bacteriome, substantial variability in HBM bacterial profiles occurs and has been linked to various exposures. For example, HBM from women who delivered vaginally showed a different bacterial profile compared to those who had caesarean section delivery (6). Other factors, such as geographical location, maternal weight and body mass index, maternal health, maternal dietary intake and lactational stage have also been shown to influence the HBM bacteriome composition (5, 6, 11-13). However, previous studies have been limited by sample size (n=10 to 133), with few studies having been performed in sub-Saharan Africa (5, 14) and studies have not yet evaluated socio-demographic and psychosocial factors in detail.

We therefore conducted a large cross-sectional study, nested within an existing birth cohort, to describe the bacteriome of HBM from a cohort of South African women and the associated factors.

5.2 MATERIALS AND METHODS

5.2.1 Study settings: Drakenstein Child Health Study

This was a cross-sectional study nested within an existing birth cohort study, the "Drakenstein Child Health Study" (DCHS) (15). The primary aim of the DCHS is to investigate the early life determinants of child health in two poor communities in South Africa, a Low and Middle-Income Country (LMIC). The birth cohort is based in the Drakenstein sub-district, a semi-rural area 60 kilometers outside Cape Town, South Africa and has an estimated population of 200,000. The study sites include the TC Newman community health clinic (which serves a population with mixed ancestry), and Mbekweni (which serves a population with predominately black African isiXhosa ancestry). These two study sites are approximately 5 km apart. Inhabitants live in informal housing or crowded conditions, with high levels of unemployment, a high prevalence of tobacco smoke exposure, alcohol misuse, malnutrition and other poverty-related exposures (15).

5.2.2 Clinical data and sample collection

Pregnant women, 18 years of age or older (n = 1137), were enrolled between 20- and 28-weeks of gestation and were followed up until their infants were 5 years old. Written informed consent was obtained from all participating mothers. On recruitment, detailed demographic and risk factor data were collected. Mothers underwent physical examination and metrics such as maternal height and weight were collected (15).

HBM samples were collected from 554 women at 6-10 weeks postpartum. All participating women were asked to wash their hands, nipples and surrounding breast area with soap and water to minimize the presence of skin bacteria. HBM was collected manually by hand expression into a sterile collection container after discarding the first few drops. After collection, the samples were transported on ice to the research laboratory at the University of Cape Town and stored at −80ºC until further processing.

Ethical approval for the present study and the parent study (DCHS) was obtained from the University of Cape Town (UCT) Human Research Ethics committee (reference numbers 649/2016 and 401/2009).

5.2.3 Bacterial nucleic acid extraction and quantification

HBM samples were homogenized by vortexing and skim milk preparation was adapted from a previously published protocol (16). In brief, the samples were centrifuged at 3500 g for 20 mins at -10 °C, and the fat layer discarded. The supernatant was then centrifuged at 7600 g for 10 mins at room temperature, and the pellet was used for DNA extraction. Total genomic DNA was extracted using the commercial manual extraction kit, ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research Corp., CA, Irvine, USA), which incorporates a bead-beading step. All bead-beating steps were performed in a TissueLyser LT (Qiagen) at a frequency of 50Hz for 5 min.

The quantity and purity of DNA was measured using a NanoDrop™ ND-2000c Spectrophotometer (Thermo Scientific, Inc.). DNA was stored at -20°C until further processing. The bacterial 16S rRNA gene was quantified using a 16S qPCR protocol previously described (17). Each 30 µl PCR reaction contained 2.5 µl DNA template, 1 µl of probe (16S-P1 (FAM- ATT AGA TAC CCT GGT AGT CCA –MGB), 15 µl Sensifast Probe No-rox (BIO-86020), 9.5 µl of MilliQ water and 1 µl each of 0.333 µM forward and reverse primer (16S-F1: 5′-CGA AAG CGT GGG GAG CAA A -3′ and 16S-R1: 5′-GTT CGT ACT CCC CAG GCG G-3′, respectively) (17). PCR cycling conditions were as follows: 50 °C for 2 mins. 95 °C for 10 mins; 45 cycles of 95 °C for 15 s, 60 °C for 60 s, and 72 °C for 1 s (17).

5.2.4 Extraction and sequencing controls

Extraction controls were processed alongside HBM specimens and were included for each batch of extractions (Appendix 2, Figure 1). Positive extraction controls consisted of 1000 µl elution buffer (provided by the ZR Fungal/Bacterial DNA MiniPrep™ kit) spiked with 75 µl Zymobiomics™ microbial community standard (Catalogue no. D6300, Zymo Research Corp., Irvine, CA, USA). Negative extraction controls (NTC; no template control) consisted of 1000 µl of un-spiked elution buffer. DNA extracts from negative extraction controls served as negative PCR and sequencing controls. A subset of DNA extracts from negative extraction controls were spiked with *Mycobacterium smegmatis* (*M. smegmatis*) DNA (Appendix 2, Figure 1B) at a 16S rRNA gene concentration similar to that obtained from HBM samples assessed by qPCR (herein referred to as "*M. smegmatis*-spiked-NTC". *M. smegmatis*-spiked-NTC were used to correct for potential contamination, resulting from bacterial DNA in buffers and reagents, during bioinformatics processing of sequenced reads. To determine experimental reproducibility, library preparation and sequencing were carried out in duplicate for 11 HBM DNA extracts. These included seven DNA extracts randomly selected for repeat processing within a single run ("within-run repeats"); and four DNA extracts randomly selected for repeat processing between the two sequencing runs performed ("between-run repeats"). In addition, the ZymoBIOMICS™ Microbial Community DNA Standard (Catalogue no. D6305, Zymo Research Corp., Irvine, CA, USA) was used to assess library preparation, sequencing reproducibility and bias.

5.2.5 16S ribosomal ribonucleic acid (rRNA) gene amplicon library preparation and sequencing

Previously published primers (18) [with slight modifications (19)], PCR conditions and library preparation steps were followed to generate 16S rRNA gene libraries (19). Libraries were constructed using a two-step amplification approach described by Wu and colleagues (20). In the first PCR reaction, the hypervariable V4 region of the 16S rRNA gene was amplified using primers (515Fshort)-5'GTGCCAGCHGCYGCGGT3' and (806Rshort)-5'GGACTACNNGGGTNTCTAAT3' respectively. Each 25.25 µl PCR reaction contained 12.5 µl 2X MyTaq™ HS Mix (BIO-25046), 2 µl each of 0.8 µM forward and reverse primers, 1 µl of MilliQ water, 0.75µl dimethyl sulphoxide (Sigma-Aldrich®, MO, USA) and 7 µl bacterial genomic DNA. PCR was carried out under the following conditions: 95 °C for 3 min; 10 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 s; and finally, 72 °C for 5 min. Amplicon product (7 µl) from the first PCR reaction was used as template in the second PCR reaction. The second PCR reaction mixture was the same as the first except for the use of modified reverse primers. Reverse primers were modified at the 3′ end with Illumina adapter sequences and unique index barcodes for each sample. The use of index sequences allowed multiplexing of samples in a single sequencing run

(18). The PCR conditions for the second PCR were the same as for the first PCR but run for 30 cycles.

Amplicon products were cleaned with the Agencourt AMPure system (Beckman Coulter, UK) using an Agencourt SPRIPlate 96 super Magnet Plate. The QuantiFluor™ dsDNA System (Promega, Madison, WI, USA) was used to quantify the cleaned amplicons. The integrity of the cleaned amplicons was assessed by gel electrophoresis. Amplicons were normalized by pooling the different samples at 100 ng each. The pooled library was purified using the Agencourt AMPure system and extracted from a 1.5% agarose gel following gel electrophoresis (30 min at 35 V; 45 min at 40 V; and finally, 3h30 min at 70 V). Excision of the pooled 16S library was followed by purification using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, United States) according to manufacturer's protocol. The elution buffer, Tris-EDTA (pH 8.0), was heated at 70 °C to improve amplicon recovery (step 13). Finally, the Qubit® dsDNA BR Assay Kit was used for quantification of the pooled library.

Sequencing was carried out at the Centre for Proteomic and Genomic Research, Cape Town, South Africa. Quality control pre-sequencing steps included quantification of adapter-ligated dsDNA using the KAPA Library Quantification Kit (Illumina®) (KAPA Biosystems, MA, USA) and analysis of the fragment size of the pooled library using the Agilent High-Sensitivity (HS) DNA Kit (Agilent Technologies, CA, USA). The library pool was diluted to 5.5 pM for sequencing and the internal sequencing control (PhiX control V3, Illumina, CA, USA) was spiked into the diluted library at 15% (v/v) according to the manufacturer's instructions (21). The first and second library pool had 352 and 202 samples multiplexed per Illumina run. The pooled 16S library was paired-end sequenced on the Illumina MiSeq® system using the MiSeq® Reagent v3 kit (600 cycles) (Illumina, CA, USA).

5.2.6 Processing of 16S rRNA gene sequences

16S rRNA gene sequences were processed using an in-house pipeline. Briefly, the sequencing quality of FASTQ files were assessed using FastQC and MultiQC packages (22, 23). Forward and reverse sequences were then merged using UPARSE (24) allowing 3 mismatches in overlaps (uparse_merge_fastq, fastq_maxdiff set to 3), followed by quality filtering using uparse_filter_fastq fastq_filter (sequences truncated to 250bp). Reads with a maximum expected error >0.1 were discarded (fastq_maxee set to 0.1). USEARCH10 (25) sortbysize command was used to dereplicate and select sequences occurring more than once. Clustering of sequences into operational taxonomic units (OTUs) (with a clustering radius of 3) was performed with USEARCH10. The USEARCH10_uchime2_ref tool was used to detect and remove chimeras, and OTU counts were obtained using USEARCH10 usearch-global.

In-silico correction for contamination of biological HBM samples was carried out using a procedure to control for "over-compensation" during contaminant removal (Appendix 2, Figure 1). Firstly, *M. smegmatis* sequences were removed from the "*M. smegmatis*-spiked-NTC" controls. Background sequences present in the *M. smegmatis*-spiked-NTC controls after the removal of *M. smegmatis* sequences were screened against biological sample sequences by aligning biological sample sequences to background sequences at 100% identity using align_seq.py, based on PyNAST (26) algorithm and uclust (25) (Appendix 2, Figure 1D). The average number of reads was calculated for each of the "background sequences" across *M. smegmatis*-spiked-NTCs, and the corresponding number of reads was thereafter removed from the biological HBM samples (Appendix 2, Figure 1E). Further processing of data was performed using Quantitative Insights Into Microbial Ecology (QIIME) 1.9.0 suite of software tools (27). Taxonomy was assigned to representative sequences of the OTUs using assign_taxonomy.py (method set to Ribosomal Database Project (RDP)) Classifier (26) and identity was set to 97% with SILVA 132 release database (28). Sequence alignment and filtering was performed with align_seqs.py (97% identity) and filter_alignment.py, while construction of a phylogenetic tree was done using make_phylogeny.py script.

Chao1 species richness (29) was used to estimate species richness and a rarefaction plot of Shannon diversity against sequencing depth was generated using alpha_rarefaction.py in QIIME 1.9.0. Samples with <1000 reads were removed from further analysis as the rarefaction curve plateaued at a sequence depth of 1000 read counts. The core bacteriome, defined as the OTUs that were present in at least 80% of the samples, was determined with QIIME with the script "compute_core_microbiome.py". Nextflow (30) was used to loop the entire processing workflow. The 16S rRNA gene sequencing datasets used in this study are stored in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) repository (BioProject PRJNA520889).

5.2.7 Statistical analyses

Statistical analysis and graphical illustrations of the data (barplots, boxplots, dendograms) were generated in R statistical package (version 3.4.4) (31) and R studio 1.1.456, respectively. Alpha diversity was calculated using the Shannon–Weaver index (32). Analysis of Variance (Type II test) (33) was used to test the difference in alpha diversity between groups, while error estimates were based on Pearson residuals.

Agglomerative clustering was performed for all OTUs with relative abundance of >0.5% and was generated by complete linkage hierarchical clustering (34) using the [hclust] function (35). This hierarchical clustering method is based on the Bray-Curtis dissimilarity index (36) of the R *vegan* package (37). To determine the relative abundances of bacterial profiles, the operational taxonomic unit (OTU) table (clustered at 97% sequence similarity) was transformed from count data to

compositional data (38). The hierarchical clustering tree was cut at a height of 0.8 to cluster samples into groups based on relative abundance of bacteria in HBM samples at genus level. The optimal number of clusters was determined by Calinski–Harabasz index (39) and validated by silhouette width index (40).

Log-ratio biplots using a Bayesian prior technique for adjustments of zero counts were made as previously described (41) and lambda-scaling was employed to ensure evenness in the "total spread" of the data sets (42). Box-plots (43) were used for visualization of distribution of the data.

A co-occurrence network was constructed using the R *network* package with centered log-ratio data (44). The cut-off was set to 0.28 to generate the network for each of the different clusters and for all samples, using bacteria with genus-level abundances >0.5%. A bivariate correlation analysis for the 16 most abundant genera was performed using Pearson correlation.

Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA) via "adonis" in the R package *vegan* (45, 46) with Aitchison's distance (47, 48) in the R package *robCompositions* (49) was used to test the effects of potential influencing factors on the composition and diversity of HBM bacteriome. ADONIS was performed with 999 x permutations and method "bray". Benjamini–Hochberg method for multiple correction was used to correct all *p*-values, set at a 5% significance level, by the false discovery rate (FDR) (50). All OTUs irrespective of their abundance at each taxonomy level were tested in the model and a multivariate p-value was generated for the covariates. Linear discriminant analysis (LDA) effect size (LEfSe) with default parameters (51) was used to asses taxa that differed between groups, for any covariate significant by ADONIS.

The Beck Depression Inventory (BDI-II) is a self-report measure of depressive symptoms that assesses key symptoms of depression between 0 (absence of symptom) and 3 (severe, often with functional impairment). Individual items are summed and scores of ≥20 is indicative of moderate/severe depression (52, 53). Self-Regulation Questionnaire (SRQ) is a WHO-endorsed self-report measure assessing psychological distress, including symptoms of depressive and anxiety disorders in which individual items are scored according to whether or not the symptom is present. SRQ scores ≥8 is indicative of "high risk" participants (54, 55). PTSD was measured by the Modified Posttraumatic Stress Disorder Symptom Scale (MPSS) (56) which is a self-report measure with scale including items which assess 3 symptom clusters for PTSD: re-experiencing, avoidance/ emotional numbing, and increased arousal. Items assessing the re-experiencing symptom cluster were scored as "above threshold" if their sum totaled ≥1; avoidance/emotional numbing ≥3; and increased arousal ≥2. Participants who scored above threshold across all three symptom clusters and reported symptom duration of at least 1 month (scored ≥1 for item 18) were classified as "suspected PTSD cases". An intimate partner violence (IPV) questionnaire was used to assess recent exposure

to physical, emotional and sexual IPV as previously described (57). The four psychosocial variables described above were collected at the 28-32 week antenatal visit.

The Alcohol, Smoking and Substance Involvement Screening test (ASSIST) was used to assess maternal alcohol consumption based on a scoring system as previously described (57). Scores of 0–10 for alcohol indicate that a participant is at low risk for substance-related health problems.

Infant length was measured in centimeters to the nearest completed 0.5 cm, and weight was measured in kilograms to the nearest completed 10 g at the hospital as previously described (58).

A coefficient of multiple determination was generated using linear regression analysis (59) to determine the reproducibility of the eleven DNA extracts which were processed in duplicate. The total read count of each OTU in a sample was compared with the total read count of each OTU in its duplicate set.

5.3 RESULTS

5.3.1 Participant characteristics

HBM samples were collected from 554 mothers at 6-10 weeks (median 7.5 weeks) postpartum. Maternal characteristics can be found as Appendix 2, Table 1. The median maternal weight and body mass index (BMI) at time of HBM collection was 63.7kg and 25 kg/m^2, respectively. The majority of the mothers were unemployed, and only 13% of participants had a household income of >ZAR5000/month (>360 USD/month). HIV prevalence was higher at the Mbekweni study site (18.4% of mothers) compared to TC Newman (2% of mothers). Smoking status, as measured by urine cotinine levels at the 28–32-week antenatal visit, classified 13% of women at Mbekweni as active smokers compared to 52% of mothers at TC Newman. At the 6-10-week postpartum study visit, only 53% of women were exclusively breastfeeding their babies. Most of the women delivered their infants vaginally (81%). Maternal post-traumatic stress disorder (PTSD) was documented in 8% of mothers, while 25% and 22% of women were above the Beck Depression Inventory (BDI) and Self-Regulation Questionnaire (SRQ) threshold, respectively. The median household size was 5 people across both sites. Twenty-three percentage (23%) of infants were delivered pre-term (Appendix 2, Table 1).

5.3.2 Sequencing results and OTU analysis

A total of 16,835,376 high-quality raw paired-end reads were obtained. The total number of post-filtered reads was 4,865,561. The median and mean sequence read count per sample was 8007 and 8782.6 (range 48-40,919) respectively**.** A rarefaction curve of Shannon diversity plateaued at a sequence depth of 1000 read counts (Appendix 2, Figure 2), therefore 32 samples with sequence read counts <1000 were removed from further analysis. USEARCH9 mapping of sequences

revealed that the sequence reads clustered into 2284 OTUs. The OTUs were classified into 58 phyla, 133 classes, 263 orders, 596 families and 1,300 genera.

5.3.3 Profiling of human breast milk bacteriome

HBM bacterial profiles revealed diverse bacterial communities. Four phyla had mean relative abundances >0.5%: Firmicutes (mean relative abundance [MRA]: 72%), Actinobacteria (16%), Proteobacteria (10%) and Cyanobacteria (0.9%) (Figure 5.1). At the genus level, 16 genera had MRAs >0.5%, of which *Streptococcus* (49%), *Staphylococcus* (18%) and *Rothia* (6%) were most abundant (Appendix 2, Figure 3). A core bacteriome consisting of 14 OTUs from 9 genera, *Corynebacterium*, *Streptococcus*, *Staphylococcus*, *Rothia*, *Veillonella*, *Gemella*, *Acinetobacter*, *Micrococcus* and a genus belonging to the family, Enterobacteriaceae, was observed (these genera were all present in HBM samples from >80% of women) (Appendix 2, Table 2).

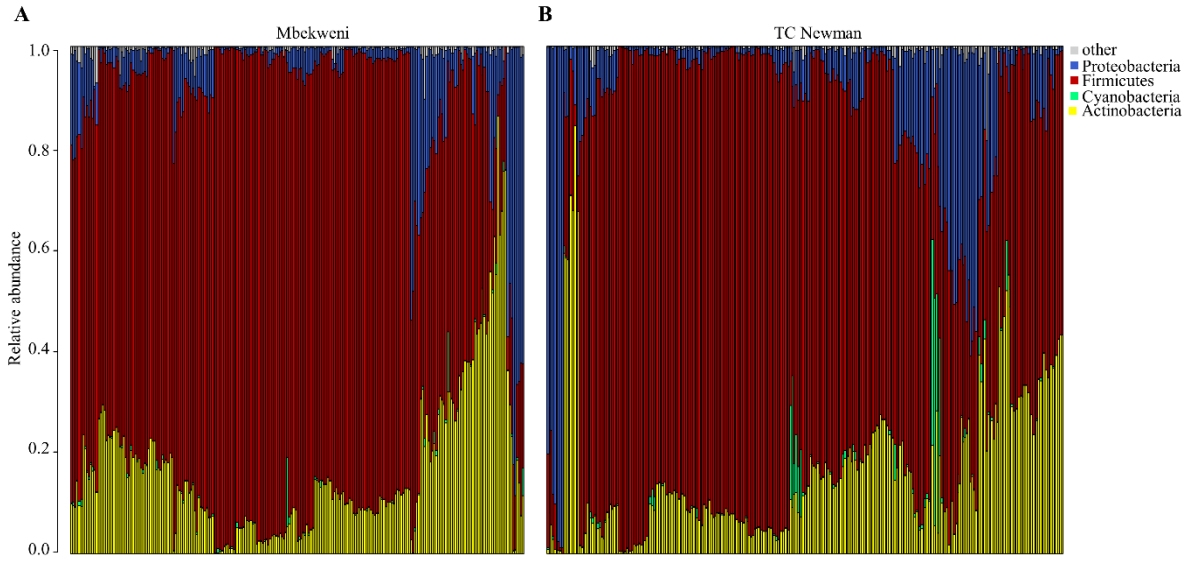

Figure 5. 1: Complete linkage unsupervised hierarchical clustering of relative abundances of human breast milk (HBM) bacterial phyla. Bar-plot of relative abundances of HBM bacteria genera at (A) Mbekweni and (B) TC Newman study sites. Each bar represents a mother's HBM bacteriome profile and each coloured box, a bacterial phylum. Phyla with less than 0.5% abundance in a given sample are grouped together, herein referred to as "other" (grey boxes).

5.3.4 Breast milk bacteriome profiles segregate into three major clusters

Hierarchical complete linkage unsupervised clustering based on Bray-Curtis dissimilarities at the genus level resulted in an eleven-cluster best fit model. Clusters 4-11 however, had very few participants and were therefore excluded from further analysis (Appendix 2, Figure 4). *Streptococcus* and/or *Staphylococcus* were responsible for the formation of the three dominant clusters in our study population. Cluster 1 showed the highest abundance of *Staphylococcus* spp., Cluster 2 was dominated by relatively equal proportions of both *Streptococcus* spp. and

Staphylococcus spp., while Cluster 3 showed the highest relative abundance of *Streptococcus* spp. (Figure 5.2). Similar numbers of HBM samples were found in clusters 1 and 2, with the majority in cluster 3 (Figure 5.2A).

Bacterial profiles of many of the most abundant taxa also differed between the cluster groups. At phylum level, Cluster 2 showed the highest abundance of Actinobacteria and Proteobacteria but lowest abundance of Firmicutes. Cluster 3, on the other hand, showed the highest abundance of Firmicutes but lowest abundance of Proteobacteria (Appendix 2, Table 3). At genus level, Cluster 1 showed the highest abundance of OTU_24 (belonging to the family Enterobacteriaceae) and *Corynebacterium,* with the lowest abundance of *Veillonella,* and *Rothia;* Cluster 2 showed the highest abundance of *Acinetobacter* and the LAB-*Lactobacillus* and *Bifidobacterium*; Cluster 3 showed the highest abundance of *Veillonella* and *Rothia* but lowest abundance of *Corynebacterium, OTU_24* and *Acinetobacter* (Figure 5.2 and Appendix 2, Table 3).

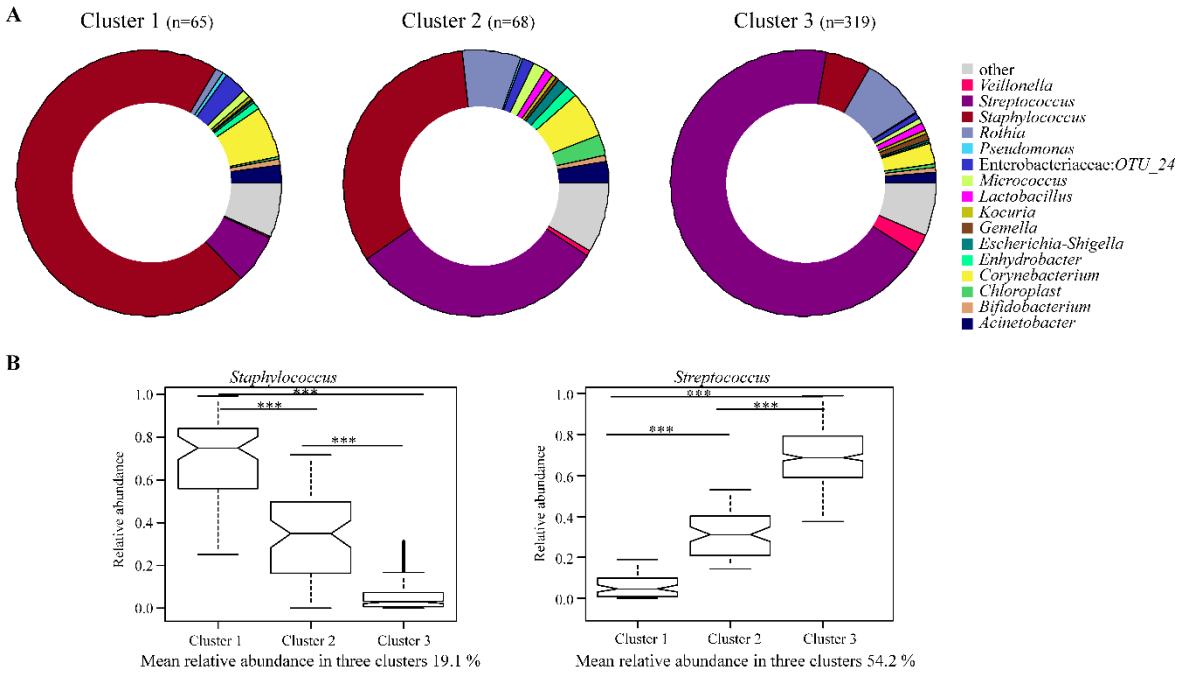

Figure 5. 2: Human breast milk (HBM) bacterial profiles of the three clusters in which samples were grouped at genus level. (A) Pie charts show the mean relative abundances in each cluster at the genus level (for genera with abundance >0.05%). (B) Notched box plots show the mean relative abundance of the predominant bacteria genera in each cluster. The notched box signifies the 75% (upper) and 25% (lower) quartile showing the distribution of 50% of the samples. The median is represented by the line inside the box plot, and the notch shows the 95% confidence interval for the median. The whiskers (top and bottom) represent the maximum and minimum values. Outliers, which are beyond 1.5 times the interquartile range above the maximum value and below the minimum value, are shown with open circles. (***$p < 0.001$).

Furthermore, exploration of β-diversity using principal coordinate analysis (PCoA) revealed distinct separation of HBM samples based on the three dominant bacterial profile cluster groups

(Figure 5.3A). PERMANOVA "adonis" analysis identified significant dissimilarity in bacterial composition between the clusters at order, family and genus taxonomy levels ($p \leq 0.001$). Cluster 3 was common at both study sites, but more prevalent at Mbekweni, while Cluster 1 was more prevalent at TC Newman ($p=0.018$) (Figure 5.3B).

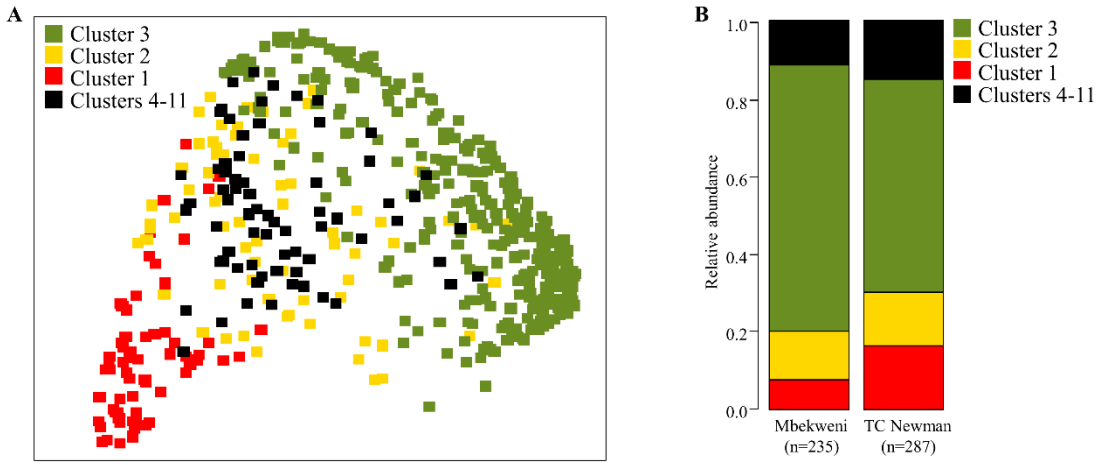

Figure 5. 3: Human breast milk (HBM) bacteriome profiles coloured by clusters. (A) Principal coordinate analysis (PCoA) of Bray-Curtis distance matrices of bacteria genera from all 522 HBM samples in the study. (B) Bar plots showing the proportion of different clusters detected from each study site.

5.3.5 Alpha diversity of bacterial communities within the DCHS cohort study

Of the 26 covariates tested in this study (Appendix 2, Table 1), only the phenotypic cluster groups and infant birth length showed significant differences in both alpha diversity measures (Shannon diversity and Chao1 index). Figure 5.4 shows the alpha diversity matrices for 5 selected covariates. For phenotypic cluster groups, cluster 1 had the lowest diversity by both measures, while Cluster 2 had the highest Shannon diversity and Cluster 3 had the highest Chao1 species richness (Figure 5.4). To analyse the association of infant birth length with HBM bacterial diversity, samples were stratified into three groups: mothers with infant birth length of 0-40cm, 40-48cm, and 48-60cm. HBM from mothers with infant birth length 48-60cm had the lowest bacterial diversity by both measures (Appendix 2, Table 4). A significant difference in Shannon diversity was observed based on maternal age in all samples and at the Mbekweni study site, with older mothers (ages ≥35) having a higher Shannon diversity index ($p<0.05$) (Figure 5.4 and Appendix 2, Table 4).

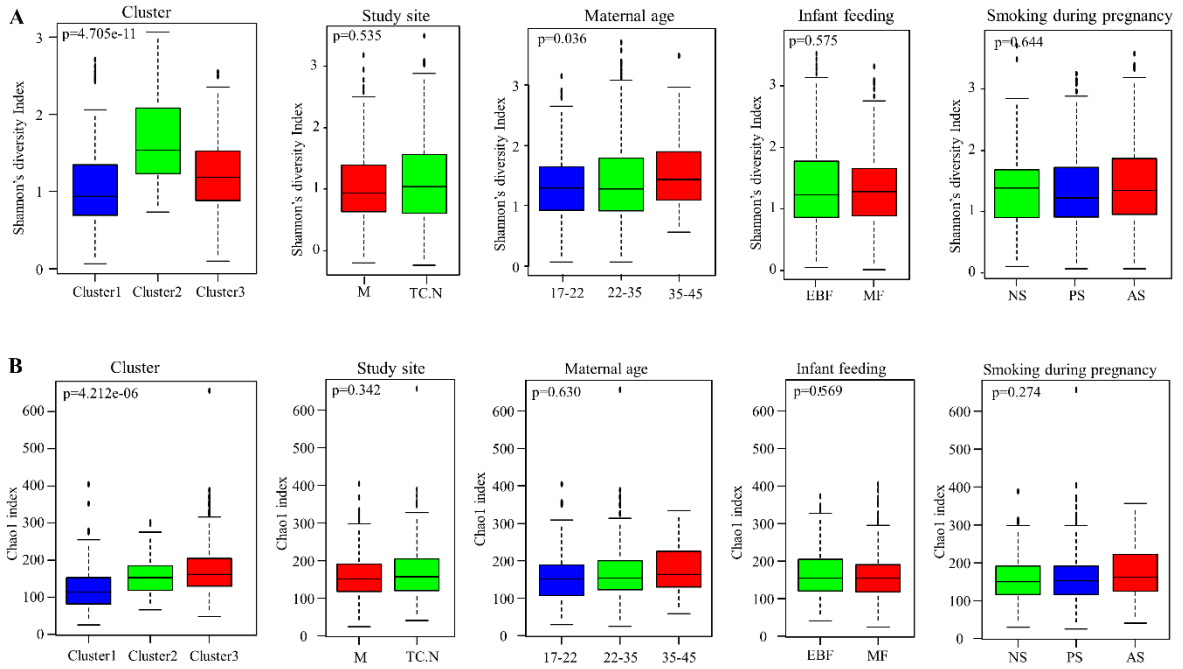

Figure 5. 4: Alpha diversity indices of human breast milk bacteriome profiles. (A) Shannon diversity index, and (B) Chao1 index of HBM bacteriome profiles of participants based on cluster group, study site, maternal age (in years), infant feeding at 6–10 weeks, and smoking during pregnancy. M = Mbekweni, TCN = TC Newman, EBF = exclusive breastfeeding, MF = mixed feeding, NS = non-smoker, PS = passive smoker, AS = active smoker.

5.3.6 Human breast milk bacterial profiles in relation to demographic, socio-economic and psychosocial variables

Beta-diversity clustering at the genus level of all HBM samples based on Bray-Curtis dissimilarity index showed no clear association between bacterial profiles and the different covariates studied (Figure 5.5). We also visualized bacterial diversity at the genus level for genera with abundances >0.5% using log ratio biplots. Clustering was not observed for any covariates studied (Figure 5.6 and Appendix 2, Figure 5). Figure 5.6 shows results for a selection of 4 covariates (study site, maternal smoking, maternal BMI and mode of delivery). Mothers with elective caesarean section delivery had bacterial profiles with less clustering towards *Staphylococcus* (Figure 6).

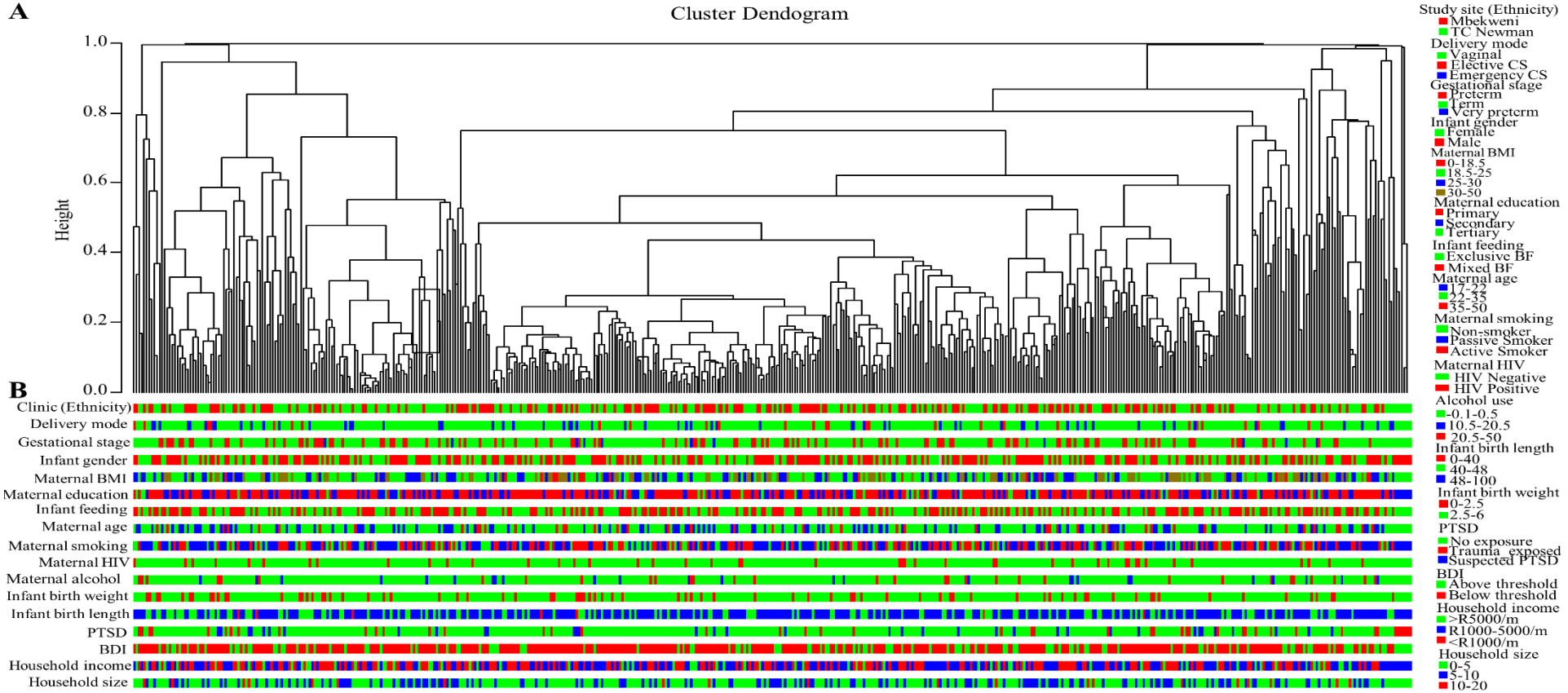

Figure 5. 5: Complete linkage unsupervised hierarchical clustering of human breast milk (HBM) bacterial profiles. (A) Dendogram of bacterial profiles present in 522 HBM samples at genus level. (B) Horizontal coloured bars below the dendogram summarize covariate data for each sample.

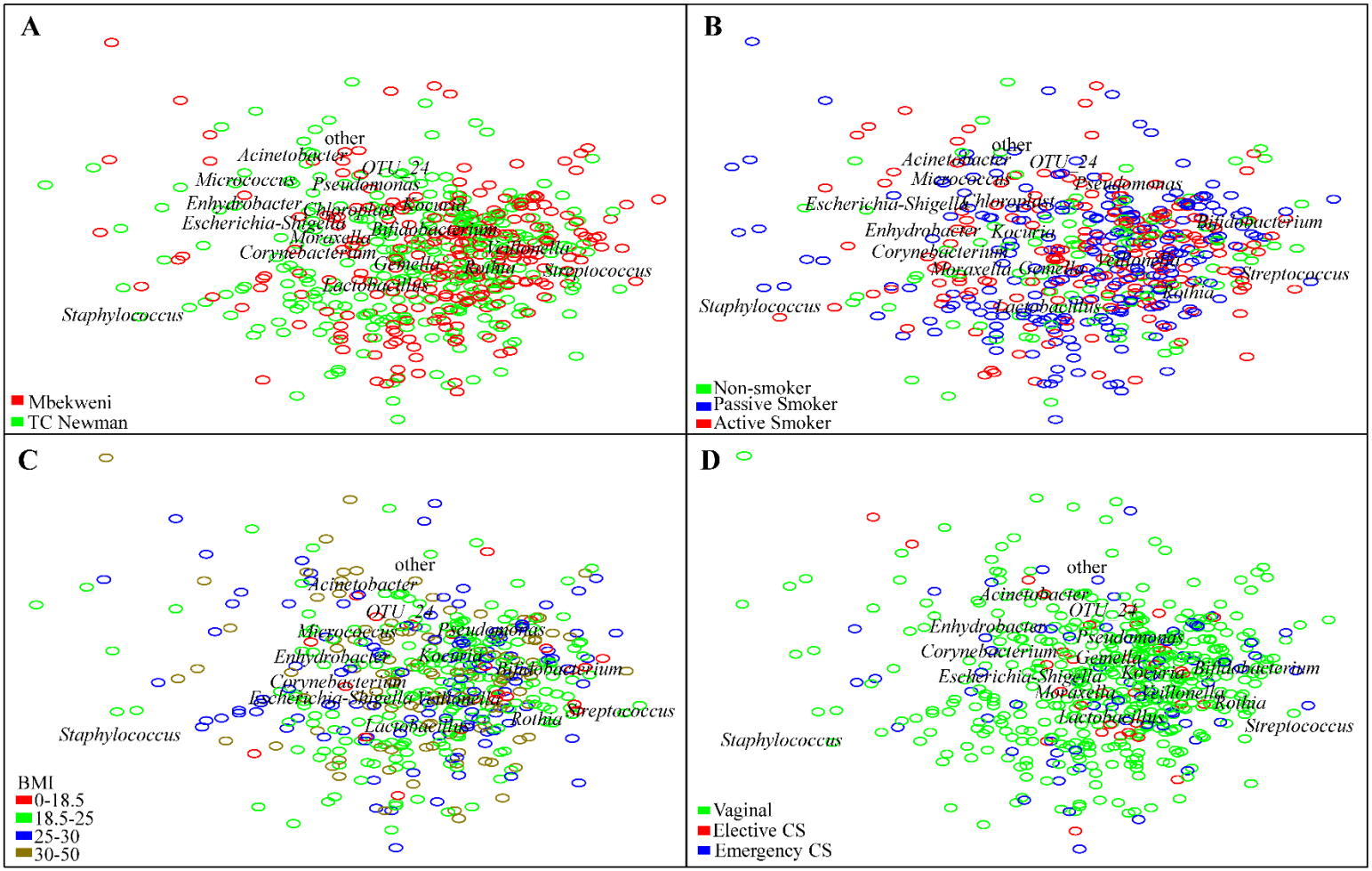

Figure 5. 6: Log ratio biplot of human breast milk bacterial abundances at the genus level. Samples are coloured according to (A) study site, (B) maternal smoking, (C) Body Mass Index (BMI), and (D) delivery mode. Each sample is represented by a circle and is coloured based on group type. Samples (circles) that cluster together are similar in bacterial composition and abundance.

Associations between bacterial profiles and socio-demographic and psychosocial characteristics were also investigated at different taxonomy levels using PERMANOVA. At the phylum, order, family and genus levels, only study site (which was also a marker for ethnicity) was associated with significant dissimilarity of the HBM bacterial communities (ANOVA, $p < 0.05$). There were no statistically significant differences between HBM bacterial profiles in relation to the remaining covariates (Table 5.1**).** LEFSe was performed to further explore differences in specific bacterial taxa in relation to study site. In line with the prevalence of the different cluster groups between study sites, HBM samples from Mbekweni had significantly higher relative abundance of the genus *Streptococcus* while samples from TC Newman had significantly higher relative abundances of the genera *Staphylococcus*, *Acinetobacter* and *Escherichia_Shigella* (Appendix 2, Figure 6A).

Table 5. 1: Permutational multivariate analysis of variance (PERMANOVA) analyses of HBM bacteriome and its association with maternal demographic and psychosocial factors.

Covariates	**Phylum (p-value)**	**Class (p-value)**	**Order (p-value)**	**Family (p-value)**	**Genus (p-value)**
Study site (Ethnicity)	0.041*	0.0628	0.0036*	0.004*	0.0028*
Mode of delivery	0.9756	0.9492	0.8276	0.8358	0.793
Gestational age	0.1266	0.126	0.944	0.3424	0.3538
Infant gender	0.7818	0.8142	0.3084	0.9608	0.7898
Infant feeding options	0.1716	0.2244	0.9502	0.2888	0.4662
Maternal education	0.275	0.3486	0.2202	0.189	0.1734
Maternal employment	0.7434	0.7192	0.2346	0.6372	0.7988
Maternal BMI	0.3886	0.3806	0.6206	0.714	0.5788
Infant birth weight	0.836	0.933	0.6886	0.719	0.7824
Infant birth length	0.9876	0.971	0.9006	0.895	0.9414
Maternal age	0.3286	0.3986	0.8976	0.641	0.7316
Dwelling type	0.5846	0.4892	0.6306	0.136	0.1158
Marital status	0.4194	0.4406	0.1362	0.7608	0.7778
Household income	0.6062	0.6988	0.663	0.0922	0.0988
Maternal HIV status	0.7238	0.6582	0.0836	0.9242	0.9252
Antibiotics	0.4178	0.4446	0.951	0.3334	0.2128
Household size	0.559	0.4248	0.3374	0.9198	0.8276
Maternal smoking	0.867	0.8698	0.837	0.7986	0.7448
Alcohol score	0.1216	0.0974	0.8012	0.2018	0.8706
IPV-emotional	0.9198	0.9132	0.806	0.8394	0.3256
IPV-physical	0.2504	0.2432	0.1708	0.6604	0.817
IPV-sexual	0.7438	0.8552	0.8676	0.2556	0.8092
PTSD	0.847	0.6846	0.724	0.3232	0.1342
BDI score	0.127	0.876	0.2598	0.3168	0.3246

SRQ	0.7408	0.5376	0.2576	0.8204	0.3012

EBF=Exclusive breastfeeding; BMI=Body Mass Index; IPV=Induced Partner Violence; PTSD=post-traumatic stress disorder; BDI=Beck Depression Inventory; SRQ=Self-Regulation Questionnaire; smoking status was based on cotinine levels measured during an antenatal visit. Cotinine levels ≥ 500 (Active smoker); Cotinine levels >10<500 (Passive smoker); Cotinine levels ≤ 10 (Non-smoker). *p*-values were generated by multivariable PERMANOVA analyses and adjusted using Benjamini–Hochberg's false discovery rate. Significant results ($p<0.05$) are labelled with asterisks.

5.3.7 Co-occurrence networks in human breast milk bacterial communities

Bacterial correlations within the HBM bacteriome were explored, since bacteria cohabit ecological niches, and interactions between bacterial species may be present (60). We constructed a network for all HBM samples, and across the 3 main clusters based on Pearson's correlations of relative abundance ($-0.28 >|r|<0.28$) at genus level (Figure 5.7 and Appendix 2, Table 5). The network of all HBM samples contained 12 nodes (bacterial genera) and 19 edges (interconnecting lines denoting correlations) (Figure 5.7A). Bacterial correlations within each cluster group were similar except for *Streptococcus* spp. and *Staphylococcus* spp. While a negative correlation was observed between these two genera in a co-occurrence network involving all samples, a positive interaction was observed in cluster 2 where the relative abundances of *Streptococcus* spp. and *Staphylococcus* spp. were similar.

Within the Firmicutes phylum, the abundance of *Staphylococcus* spp. was positively correlated with *Corynebacterium* spp. (both common commensals of the skin), but was negatively correlated with *Streptococcus* spp. and *Veillonella* spp. *Streptococcus* spp. abundance, on the other hand, was positively correlated with other members of the oral flora, *Veillonella* spp., *Gemella* spp., and *Rothia* spp., and negatively correlated with the Proteobacteria (*Acinetobacter* spp., and *Enhydrobacter* spp.), and with skin commensals such as *Micrococcus* spp. and *Staphylococcus* spp. A positive correlation was observed between *Pseudomonas* spp., *Acinetobacter* spp. and *Enhydrobacter* spp., all members of the order Pseudomonadales.

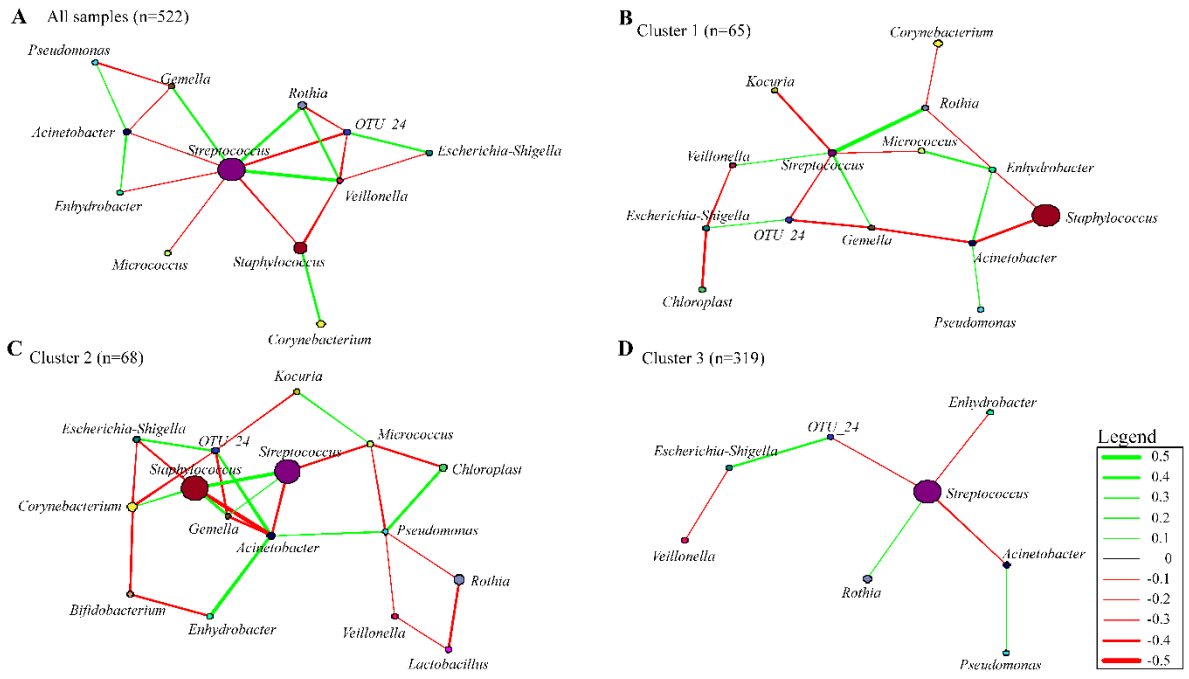

Figure 5. 7: Bacterial networks within the human breast milk bacteriome community based on correlation between relative abundances. Figures show correlations between predominant bacteria in (A), all HBM samples, (B), Cluster 1, (C), Cluster 2, and (D), Cluster 3. Nodes and node sizes represent bacterial genera and their relative abundances, respectively. Green and red lines represent positive and negative correlations respectively between the bacteria, with the thickness of the line indicating the degree of correlation.

5.3.8 Reproducibility of bacterial profiling

The bacterial composition of each sample and its repeat was evaluated to test for reproducibility. Multiple R-square values of >0.97 for both within-run and between-run repeats showed excellent reproducibility (Appendix 2, Figure 7). Hierarchical clustering also showed that replicate samples clustered together (Appendix 2, Figure 8).

5.4 DISCUSSION

In this study, the largest of its kind to date, we describe the composition of the HBM bacteriome in samples from mothers living in South Africa. We identify a core bacteriome consisting of 9 bacterial genera present in >80% of samples. We also show that there are three major HBM bacteriome profiles, distinguished by the relative abundance of the genera *Streptococcus* and *Staphylococcus* and that bacteria which are commonly found as part of the skin flora correlate in relative abundance in HBM, as do bacteria which are part of the oral microbiota. We are able to demonstrate an association between study site (a proxy for ethnicity in this study), infant birth length and maternal age, a range of potential determinants and the composition of the HBM bacteriome.

Previous studies of the HBM bacteriome have revealed a diverse bacterial population, including gram positive (*Corynebacterium* spp., *Lactobacillus* spp., *Bifidobacterium* spp., *Staphylococcus* spp.) and gram negative (*Pseudomonas* spp., *Veillonella* spp.) bacteria (7, 9, 61, 62). The most abundant bacterial phyla in our study were Firmicutes and Actinobacteria, in contrast to several other studies (8, 9, 63-65) which described a predominance of Proteobacteria and Firmicutes. Our findings are similar, however, to those of Williams et al (13) who showed similar relative abundances at the phylum level in HBM from mothers in the U.S.A. Many factors including geographical region (which influences diet and cultural practices) (66), lactational stage, and maternal health may influence the HBM bacterial community, with considerable diversity reported between individuals (5, 63). Methodological differences including sample processing and analytical techniques may also account for differences between studies.

At the bacterial genus level, *Streptococcus* spp. and *Staphylococcus* spp. were most abundant, in line with previous studies (7-9, 63, 67, 68) and a recent systematic review (69). Other abundant genera included *Rothia* spp., *Corynebacteria* spp. and *Acinetobacter* spp. Of note, the relative abundance of *Acinetobacter spp.* observed in our cohort (2.2%) was low. When comparing our findings to those published previously; we found that studies with sample collection protocols similar to ours identified *Acinetobacter* spp. proportions comparable to those in our study (67). In contrast, proportions of *Acinetobacter* spp. were higher (32%) when the sample collection protocol omitted cleaning of breast skin prior to collection and discarding of the first few drops of milk during the collection procedure (67).

Bifidobacterium spp. and *Lactobacillus* spp. [lactic acid bacteria (LAB)] were detected at low relative abundances (mean relative abundances of 1.0% and 0.9%) but detected in 72% and 76% of women in our study. Previous studies using quantitative polymerase chain reaction (qPCR) have estimated the absolute abundance of *Lactobacillus* spp. and *Bifidobacterium* spp. as 10^3–10^4 cells/ml and 10^2–10^5 cells/ml respectively (4, 70, 71), however, we are not able to determine absolute abundance using 16S rRNA gene amplicon sequencing. LAB have been identified as probiotics which colonize the infant gut even at relatively small dose, and function to competitively exclude pathogens (72, 73). Studies have suggested that HBM is an important source of *Bifidobacterium* for the infant gut. In support of this, it has been shown that *Bifidobacterium* dominates the gut of a breastfed full-term infant as early as the first 3-6 days of life and makes up 60-90% of the total bacteriome of the infant gut (74). Furthermore, identical strains of *Bifidobacterium breve* and *Lactobacillus plantarum* have been isolated from HBM and infant feces in a mother-infant pair, confirming vertical transmission (9).

Our study confirmed the existence of a shared and conserved “core” bacteriome in HBM which is consistent with previous studies (7-9, 64, 65, 75). The core bacteriome identified from HBM samples assessed in our study included eight bacterial genera at 80% prevalence. *Bifidobacterium* and *Lactobacillus* were not part of the core bacteriome in our study, in contrast to a previous study in Ireland (9).

Further investigation into HBM bacterial profiles using clustering analysis revealed three major dominant profiles present in our samples, determined by the relative abundances of *Streptococcus* spp. and/or *Staphylococcus* spp. Similar to our study (76), the cluster dominated by *Staphylococcus* spp., was associated with a less diverse bacteriome. Previous studies among lactating women in Italy, and Taiwan and mainland China similarly described three bacterial profiles with clustering of bacterial profiles based on the dominance of *Streptococcus* spp. or *Staphylococcus* spp., either at the genus and family taxonomy level respectively (63, 76). A Canadian cohort has however described four clusters based on the relative abundances of a different set of bacterial profiles including Moraxellaceae, Enterobacteriaceae, and Pseudomonadaceae (Cluster 1), Streptococcaceae, Staphylococcaceae, and Oxalobacteraceae (Cluster 2), Oxalobacteriaceae and Comamonadaceae (Cluster 3), and Streptococcaceae and Comamonadaceae (Cluster 4) (65).

5.4.1 Bacterial interactions within the human breast milk bacterial community

We explored the correlation between bacterial genera, based on relative abundance. We showed a positive relationship between the two skin commensals, *Staphylococcus* spp. and *Corynebacterium* spp. in line with previous findings by Ma et al. (2015) (77). Recently, an "evil alliance" was proposed to occur between these two genera, as both have been implicated as the cause of mastitis in breastfeeding women (77, 78). *Staphylococcus epidermidis* and *Staphylococcus aureus* are major causes of infectious mastitis, a major reason of premature cessation of breastfeeding among lactating women (1). *Corynebacterium* spp., has been implicated as the third most prevalent bacterial group causing infectious mastitis (78).

Veillonella spp., *Gemella* spp., *Rothia* spp. and *Streptococcus* spp. which are commensals of the oral cavity, demonstrated positive correlations. A similar positive correlation was also observed between *Pseudomonas* spp., *Acinetobacter* spp. and *Enhydrobacter* spp. which are all members of the order Pseudomonadales and live in the nasal cavities.

A negative correlation was observed between the abundances of *Acinetobacter* spp. and *Staphylococcus* spp. Unlike a previous study in Taiwan and Mainland China (63), Correlations between *Streptococcus* spp. and *Staphylococcus* spp. varied between cluster groups, being negative for clusters 1 and 3, but positive for Cluster 2 where both bacterial genera had high relative abundances.

5.4.2 Impact of maternal and infant factors on human breast milk bacterial profiles

We studied a broad range of potential influencing factors of the HBM bacteriome but showed no association of maternal, socio-economic and psychosocial variables with the HBM bacteriome, apart from maternal age, infant birth length and study site (which almost completely correlated with ethnicity). Our findings are similar to those of Urbaniak and colleagues (2016) who found that HBM bacterial profiles did not differ significantly based on mode of delivery, gestation or infant gender in a Canadian population (8). In support of our and Urbaniak and colleagues' findings, a previous study conducted on HBM samples among

Chinese women (n=90), and a longitudinal study conducted among American women (n=104) also found that the mode of delivery had no influence on HBM bacteriome (13, 67).

In contrast to these reports, two recent studies show separation of HBM bacterial profiles on principal component analysis of beta diversity in relation to mode of delivery (64, 79). Both studies, however, had relatively small sample sizes (12 and 84 participants). In our study, we observed separation of HBM bacterial profiles of women who underwent elective caesarean section from HBM profiles of women who underwent either vaginal or emergency caesarean section delivery in a log ratio biplot with less clustering around *Staphylococcus*, however this association did not show statistical significance on PERMANOVA testing. Kumar et al. (2016) showed that while delivery mode was not associated with HBM bacterial profiles among South African and Finnish women, Chinese and Spanish women showed different bacterial profiles based on mode of delivery (5). Li et al (2017) found no difference in the abundance of bacterial families in HBM based on delivery mode, however, the genus, *Lactobacillus*, differed between the two groups (63). In another recent study, mode of delivery was associated with bacterial composition of colostrum. Higher abundances of *Pseudomonas* spp., *Staphylococcus* spp. and *Prevotella* spp. were observed in the colostrum of mothers with caesarean section delivery. It is possible that differences in HBM profiles would be most marked in early HBM samples where the hormones involved in the delivery process still have an impact.

Other factors which have been associated with HBM bacterial profiles include gestational age, maternal age, and maternal BMI. Interestingly one previous study, using qPCR showed differences in HBM composition between preterm and term milk, with higher *Bifidobacterium* spp. and lower *Enterococcus* spp. counts among women who delivered term babies (71). A more recent study and ours, using 16S rRNA gene sequencing, showed no impact of gestational age on the HBM bacteriome (8). With focus on infant gender, a previous study reported higher mean relative abundances of *Streptococcus* spp. and lower mean relative abundances of *Staphylococcus* spp. from HBM of mothers with male infants (13), although we did not find any significant association. Contrary to our study, BMI within the normal range was associated with higher proportions of Alphaproteobacteria and Betaproteobacteria at the class level (80). Maternal age, on the other hand, influenced the diversity of HBM bacterial profiles in our study and previous reports (63, 80). How maternal age or infant sex drives the bacteriome is largely unknown till date.

A study in Taiwan showed no influence of BMI on HBM bacteriome (63), as was found in our study, however, higher *Granulicatella* spp. relative abundance was observed in another study of HBM from overweight and obese mothers (13). A more recent study showed that maternal obesity was inversely associated with Proteobacteria diversity but positively associated with Firmicutes diversity at the phylum level (65).

There is increasing awareness of the microbiota-gut-brain axis, with several studies describing an association between the gut microbiota, neuropsychiatric and psychosocial variables in both mouse models

and humans (81-83). We however did not observe any association between psychosocial variables and the HBM bacteriome. To the best of our knowledge, this is the first study to examine the association of psychosocial variables with HBM bacteriome composition.

In our study, we found an association between study site and the HBM bacteriome. However, since ethnicity was almost completely correlated with study site, we are unable to determine whether these differences were due to ethnicity or other unmeasured factors associated with study site. Li et al (63) demonstrated similar findings in a study characterizing the HBM bacteriome from various regions within Taiwan and Mainland China (63). Ethnicity has been found to have an influence on the gut microbiome in several studies (66, 84), and could be driven by a range of factors, such as genetic differences or diet (85).

Strengths of our study include large sample size, consistent methods for sample collection, processing and sequencing, detailed metadata collection, a reproducible sequencing pipeline and robust multivariate statistical analysis. The major limitation of our study was the use of a single sampling time point, which precludes us from studying intra-individual variability or exploring changes in bacterial profiles and associated risk factors over time.

5.5 CONCLUSION

We used 16S rRNA gene sequencing to characterize the HBM bacteriome of a large cohort of mothers living in South Africa. We showed that the HBM bacteriome was dominated at the phylum level by Firmicutes and Actinobacteria, and at the genus level by *Staphylococcus*, *Streptococcus* and *Rothia*. We identified three major microbiome profile groups, defined by the relative abundances of *Staphylococci* and *Streptococci*. We found little evidence of the association of various socio-economic or psychosocial variables with the HBM bacteriome, but we showed that maternal age, infant birth length, and study site were associated with composition of the HBM bacteriome.

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CHAPTER 6

HUMAN BREAST MILK BACTERIAL DYSBIOSIS IS ASSOCIATED WITH LACTOSE FERMENTATION AND POOR BREAST-FEEDING OUTCOMES

Abstract

Human breast milk (HBM) is universally regarded as the optimal source of nutrition for the growing infant, however the impact of variation in the different components of HBM on breastfeeding outcomes is not fully understood. In particular, the relationship between the HBM metabolome and microbiome has not been well-studied. Here we show that a dysbiotic HBM microbiota is associated with evidence of fermentation of lactose in HBM, low lactose concentration and poor breastfeeding outcomes. We identified a subset of 45/519 (8.7%) South African lactating mothers with markedly reduced HBM lactose concentrations. Mothers with low-lactose (vs normal lactose) HBM had reduced median duration of exclusive breastfeeding (27.6 vs. 55.2 days) and their infants grew less well during the period of exclusive breastfeeding (reduced weight and length z-scores). Metabolomic profiling of low-lactose HBM revealed an increase in metabolites associated with mixed acid fermentation (characteristic of microbial carbohydrate metabolism) and depletion of metabolites in the tricarboxylic acid cycle. We therefore explored the bacterial composition of HBM using 16S rRNA amplicon sequencing and showed that the bacterial composition in samples with low lactose was markedly different from those with normal lactose. Low-lactose HBM had a strikingly higher median relative abundance of *Staphylococcus* species (19.0% vs. 5.0%), lower abundance of *Streptococcus* species (32% vs. 58%) and increased bacterial load. Since the HBM microbiota is a potentially modifiable risk factor, this may provide opportunity for diagnostic and therapeutic intervention to avert poor breastfeeding outcomes. These findings should be explored in other populations to identify women and their infants at risk.

6.1 INTRODUCTION

Human breast milk (HBM) is a complex physiological fluid universally regarded as the optimal source of nutrition for growing infants (1-3). It consists of essential nutrients including fatty acids, carbohydrates and protein, a host of non-nutritive bio-molecules including glycoconjugates, oligosaccharides, and anti-microbial peptides, and a resident microbial flora (4, 5). Despite the importance of HBM in infant health (6), the relationship between the HBM metabolome and bacteriome has not been comprehensively characterized in large studies, and related to infant health and growth.

6.2 MATERIALS AND METHODS

6.2.1 Subjects and sample collection

This cross-sectional study was nested within a birth cohort study, the "Drakenstein Child Health Study" (DCHS) which aims to investigate the early life determinants of child health within the Drakenstein sub-district in South Africa (7). Pregnant women, 18 years of age or older (n = 1137), were recruited antenatally from clinics within the Drakenstein sub-district, Western Cape, South Africa between 20- and 28-weeks' gestation and were followed up until their infants were 5 years old. On recruitment, detailed demographic and risk factor data were collected (7). HBM samples were collected from 554 women at 5-10 weeks postpartum. To ensure a narrow collection window, HBM samples collected before 5 weeks and after 10 weeks (n=35) were excluded from further analysis. None of the women described difficulties with breastfeeding at the time of breast milk collection. Women were asked to wash their hands, nipples and surrounding breast area with soap and water. Milk was collected manually by hand expression into a sterile collection bottle after discarding the first few drops. After collection, the samples were immediately refrigerated at 4 ^{o}C for a maximum of 4 hours, then transported on ice to the laboratory and then stored at −80 ^{o}C until further processing. Ethical approval for the present study and DCHS study was obtained from the Human Research Ethics committee of the University of Cape Town (reference numbers 649/2016 and 401/2009).

6.2.2 Demographic and growth measures

Socio-demographic and health information were obtained from questionnaires administered to mothers during antenatal and postnatal study visits. Birthweight and length were measured at the hospital. Gestational age at birth was estimated based on an antenatal ultrasound carried out in the second trimester. Trained study staff collected serial anthropometric measurements on the infants across 8 different time points (weeks^{+days}: 5^{+0}-8^{+6}, 9^{+0}-11^{+6}, 12^{+0}-19^{+6}, 20^{+0}-34^{+6}, 35^{+0}-45^{+6}, 46^{+0}-70^{+6}, 71^{+0}-95^{+6}, 96^{+0}-120^{+6}). Infant length was measured in centimetres to the nearest completed 0.5 cm, and weight was measured in kilograms to the nearest completed 10 g. Maternal height was measured to the nearest 0.1 cm at enrolment. BMI was calculated using weight in kilograms divided by length in centimetres squared. All anthropometric measurements were carried out twice for each child, with a third measurement if the difference between the first and second measurement differed

by more than 0.5 cm for length or more than 0.5 kg for weight. Birth weight, length, and BMI were converted to z-scores for gender and gestational age using the INTERGROWTH-21st standards (8). Infants were classified into low birthweight (LBW) if they weighed less than 2500g at birth, preterm if born at less than 37 weeks of gestational age and small for gestational age (SGA) if weighted less than the 10th percentile for gestational age, based on gender and gestational age. Corrected gestational age was used to calculate the WHO z-score for preterm infants. The weight and length measurements following birth were converted to z-scores based on age and gender, using Anthro software (9). Weight-for-age z-scores (WFAZ), height-for-age z-scores (HFAZ) and BMI for age z scores (BMIZ) were calculated.

Infant feeding practice was categorized based on the World Health Organization's (WHO) Infant and Young Child feeding indicators, namely exclusive breastfeeding or mixed feeding, which included infant cow milk-based formula with or without breastmilk or semisolids (10).

Validated questionnaires were administered to mothers at the 28-32 week antenatal visit to assess psychosocial factors. Beck's Depression Inventory is a self-report measure of depressive symptoms that assesses key symptoms of depression between 0 (absence of symptom) and 3 (severe, often with functional impairment). Individual items are summed and ≥ 20 is indicative of moderate/severe depression (11, 12). The Self-Regulation Questionnaire (SRQ) is a WHO-endorsed self-report measure assessing psychological distress in which individual items are scored according to whether or not the symptom is present; score ≥ 8 is indicative of "high risk" participants (13, 14). Post-traumatic Stress Disorder (PTSD) was measured by the Modified PTSD Symptom Scale (MPSS) (15) which is a self-report measure with scale including items which assess 3 symptom clusters for PTSD. An intimate partner violence (IPV) questionnaire was used to assess recent exposure to physical, emotional and sexual IPV as previously described (16). Exposure was categorized as above the threshold if a participant reported more than one incident of IPV during the previous year. Peritraumatic Distress Inventory (PDI) is a self-report measure assessing the level of distress experienced during and immediately after a traumatic event with each item that assesses symptoms of distress related to the event scored between 0 ("not at all") and 4 ("extremely") (17).

The Alcohol, Smoking and Substance Involvement Screening test (ASSIST) was used to assess maternal alcohol consumption based on a scoring system as previously described(16). Scores of 0–10 for alcohol indicate that a participant is at low risk for substance-related health problems. Tobacco smoke exposure was measured using maternal urinary cotinine levels at antenatal visit. A urinary cotinine level of ≥500 ng/mL was used as the threshold to categorize mothers into active smokers. Maternal diet was assessed at 6 weeks postpartum with a validated food frequency questionnaire (18).

6.2.3 Human breast milk sample preparation

HBM samples were homogenized by vortexing and skim milk was prepared using an adaptation of a previously published protocol (19). In brief, 1 ml of each sample was centrifuged at 3500 g, -10 °C for 20

mins to remove the fatty layer. The remaining sample was thereafter centrifuged at 7600 g for 10 mins at room temperature to sediment the pellets. 800 µL of sample supernatant was collected into tubes and stored at -80 °C for NMR processing, whilst pellets were stored at -80 °C for bacterial profiling.

NMR experiments were carried out at the Centre for Human Metabolomics, Potchefstroom, South Africa. Samples were filtered using a pre-rinsed 2ml Amicon® Ultra 10 kDa centrifugal filter unit (Merck; Ref UFC201024) by centrifuging at 4500 *g* for 30 mins to remove residual lipids and protein. The filter was pre-rinsed twice with distilled water by centrifugation at 4500 *g* for 15 mins to remove trace amounts of glycerol and glycerin from membrane filters. A 60 µl volume of NMR buffer solution (pH 7.4) was added to 540 µL filtrate in a microcentrifuge tube. The NMR buffer solution consisted of 1.5 M potassium phosphate buffer (pH 7.4) in deuterium oxide and 5.805 mM of trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP; Sigma-Aldrich, St. Louis, MO, USA). A trace amount of sodium azide was included in the buffer to prevent bacterial growth in the sample. A 600 µL filtrate-buffer solution was mixed under vortex to ensure complete homogeneity and transferred to a 5 mm NMR tube (Wilmad Royal Imperial; Wilmad LabGlass, USA). A total of 6 samples were excluded from the analysis because their sample preparation did not reach the standard of quality.

6.2.4 Nuclear magnetic resonance (NMR) profiling

Samples were measured at 500 MHz on a Bruker Avance III HD NMR spectrometer (Bruker Analytische Messtechnik, Karlsruhe, Germany) equipped with a triple-resonance inverse (TXI) ^{1}H probe head and x, y, z gradient coils. ^{1}H NMR spectra were acquired as 128 transients in 32K data points with a spectral width of 6002 Hz and acquisition time of 2.72 sec (relaxation delay time of 4s; 8µs (90°) pulse; sample temperature 300 K). Receiver gain was set to 64 and water suppression was by single frequency irradiation focused on H_2O resonance. Shimming of the samples was performed automatically on the deuterium signal. The resultant raw spectral data were Fourier transformed. These transformed spectra were automatically corrected for phase and baseline using Bruker Topspin (V3.5) (Bruker Biospin, Germany). Spectra were calibrated in automation to the TSP signal (0.00 pm). Spectral regions belonging to water (4.70–5.00 ppm) and urea (5.68–5.96 ppm) were removed from subsequent analysis. Spectral analysis was by "fixed-width" binning - dividing the spectra into set widths of 0.02 ppm.

Identification of signals was undertaken using the SBASE database in Amix (v3.9.11; Bruker BioSpin, Germany) or available assignments in the literature (20). A commercial spectral library from Bruker, along with pure compound spectral libraries of oligosaccharides (Elicityl-OligoTech®, France), were used to identify HMO signals. In addition, two-dimensional analysis (JRES and COSY) of pooled HBM samples was done to confirm metabolite identification. The peaks of the identified metabolites (Appendix 3, Table **6**) were fitted by a combination of Voigt functions based on the multiplicity of the NMR signal (21) using an in-house R script. The absolute concentration of each metabolite was calculated according to the equation described by

Serkova et al (22). To consider the variability introduced by the preparation of the NMR samples, the area of the spectra was corrected using the area of TSP (Appendix 3, Figure 1A) with the following formula:

$N_i = 1 - (TSP_i \times 0.1 / TSP_{median})$

Where N is the coefficient multiplied to i^{th} spectrum, TSP_i is the area of the TSP signal of the i^{th} spectrum, and TSP_{median} is the median value of the TSP signals.

Pooled supernatant from all HBM samples was used as a quality control sample and was included in each batch for qualitative assessment of repeatability by overlaying the raw spectra. No notable deviations were detectable (Appendix 3, Figure 1B). We did not detect any deviations in the lactose concentration among the quality control samples (Appendix 3, Figure 1C).

The HMO phenotype of each sample was assigned according to the presence of 3'FL, 2'FL and LNDFH I HMOs as described in the Appendix 3, Figure 2.

6.2.5 Human breast milk bacterial profiling by 16S rRNA amplicon sequencing

Total genomic DNA was extracted from pellets using the ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research Corp., Irvine, USA), which incorporates a bead-beating step. DNA yield was assessed using a 16S qPCR protocol previously described (23). PCR amplicon libraries were constructed using a two-step amplification approach described by Wu and colleagues (24) to avoid PCR amplification biases associated with the use of adapter and index sequences. The hypervariable V4 region of the 16S rRNA gene was amplified as previously described (25) with primers containing Illumina adapters and various unique index sequences at the 3' end for each sample (26). Amplicons were normalized by pooling at an equimolar concentration of 100 ng, and purified using the Agencourt AMPure system (Beckman Coulter, UK). Excision of the pooled 16S library was followed by purification using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, United States). The Qubit® dsDNA BR Assay Kit was used for final quantification of the pooled library. The quantitation of adapter-ligated dsDNA and analysis of fragment size of the pooled library was done with the KAPA Library Quantification Kit (Illumina®) (KAPA Biosystems, MA, USA) and Agilent High-Sensitivity (HS) DNA Kit (Agilent Technologies, CA, USA), respectively. The library pool was diluted to 5.5 pM for sequencing and the internal sequencing control (PhiX control V3, Illumina, CA, USA) was spiked into the diluted library at 15% (v/v)(27). The pooled 16S library was paired-end sequenced on the Illumina MiSeq® system using the MiSeq® Reagent v3 kit (600 cycles) according to the manufacturer's instructions (27). Reagents used for DNA extraction and PCR amplification were also sequenced as no template controls.

6.2.6 Processing of 16S rRNA gene sequences

The quality of FASTQ files was assessed using FastQC and MultiQC packages (28, 29). Forward and reverse sequences were then merged using UPARSE (30) allowing 3 mismatches in overlaps (uparse_merge_fastq,

fastq_maxdiff set to 3), followed by quality filtering using uparse_filter_fastq fastq_filter (sequences truncated to 250bp). Reads with a maximum expected error >0.1 were discarded (fastq_maxee set to 0.1). USEARCH10 (31) sortbysize command allowed for dereplication and selection of sequences occurring more than once. Clustering of sequences into operational taxonomic units (OTUs) (with a clustering radius of 3) was performed with USEARCH10. The USEARCH10_uchime2_ref tool was used to remove chimeras, and OTU counts were obtained using USEARCH10 usearch-global. OTU picking was at 97% sequence identity and taxonomic assignment was carried out using assign_taxonomy.py (method set to Ribosomal Database Project (RDP)) Classifier (32) and identity was set to 97% with SILVA 132 release database (33) in Quantitative Insights Into Microbial Ecology (QIIME) 1.9.0 suite of software tools (34). The dataset supporting the bacteria profiling results of this article is available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) repository (BioProject PRJNA520889).

6.2.7 Statistical and data analysis

Statistical analysis and graphical illustrations of the data were generated in R statistical package (version 3.4.4) (35) and R studio 1.1.456 using scripts developed in-house. The Iglewicz and Hoaglin outlier test (36) was used to classify the HBM samples into low and normal level of lactose based on the lactose concentration. *P* less than 10^{-3} was considered significant.

Wilcoxon Rank Sum test (37) and Kruskal-Wallis Rank Sum Test (38) were used to compare differences in numerical covariates (e.g., maternal age). Fisher's exact test (39) was used to assess differences between categorical variables (e.g., ethnicity). Kaplan–Meier plot (40) was used to visualize the difference of the percentage of exclusive breastfeeding between low and normal lactose level across time. The Wald test was used to calculate the statistical significance (*P*) of the differences between curves. Spearman rank correlation was used to explore the relationships of lactose across time postpartum. PCA was performed on the processed spectra to obtain an overview of the structure of the data. The KODAMA algorithm was used to facilitate identification of patterns representing underlying metabolic phenotypes on all samples in the data set. A fuzzy version of the known k-means clustering algorithm (41) was used to identify the different bacterial signatures in the KODAMA plot. Only entries with membership grades higher than 60% were considered in the classification of the bacteria signatures. Dendrograms were performed using Euclidean distance and Ward linkage. $P<0.05$ was considered to be significant. To account for multiple testing, a false discovery rate (FDR) of <20% was applied (42).

6.3 RESULT AND DISCUSSION

6.3.1 Participant characteristics

Here, we characterized HBM samples from 519 women enrolled in a birth cohort study, the Drakenstein Child Health Study (7). The socio-demographic features of the study participants are shown in Appendix 3, Table 1.

Many participants are poor with 41% of women having a mean household income of less than US$70 per month. HIV infection among mothers was low (9%). Each woman donated a HBM sample between 5 and 10 weeks postpartum.

6.3.2 Metabolic profiles of human breast milk (HBM) cluster according to lactose level and HMO phenotype.

We first used Nuclear Magnetic Resonance (NMR) spectroscopy to identify lactose (Figure 6.1) and characterize the specific pattern of fucosylated human milk oligosaccharides (HMOs) of each sample (Figure 6.2A). The fucosylation pattern of HMOs correlates with the activity of the enzymes, α1,2 fucosyltransferase (FUT2) and α1,3/4 fucosyltransferase (FUT3), encoded by the Secretor gene (*Se*) and the Lewis gene (*Le*) respectively (43, 44) (Figure 6.2B). We identified HMO phenotypes (i.e., Se+/Le+, Se-/Le+, Se+/Le- and Se-/Le-) based on the presence of 3′ fucosyllactose (3′FL), lacto-N-fucopentaose I (LNFP I), and lacto-N-difucohexaose I (LNDFH I) in the NMR spectra of HBM samples (Figure 6.2B). Se+/Le+ was the largest group, with 310 women, followed by Se-/Le+, Se+/Le- and Se-/Le- phenotypes with 120, 84 and 5 women respectively (Appendix 3, Table 1).

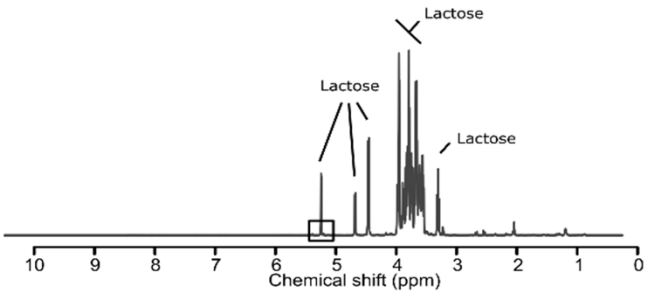

Figure 6. 1: ^{1}H-NMR spectrum of a representative HBM sample. Representative HBM sample showing NMR signals of lactose.

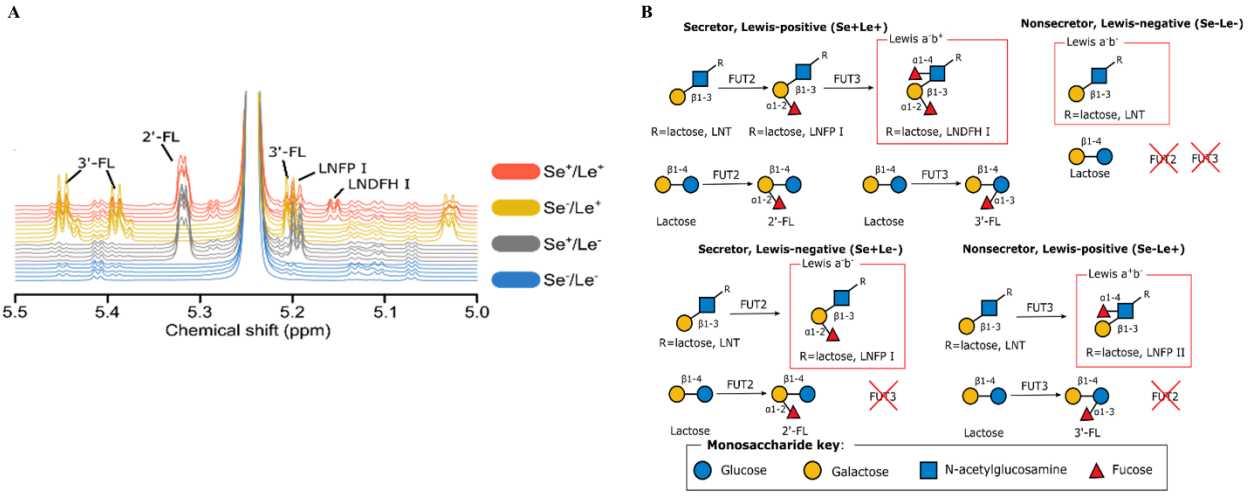

Figure 6. 2: Distinct metabolic profiles of human breast milk (HBM) are observed based on HMO phenotype. (A) HMO phenotype detection using the NMR signals of 3'-FL, 2'-FL and LNDFHI. (B) Graphical representation of the enzymatic processes that lead to the different HMO phenotypes. The figure was adapted from Bode 2012 (45). FUT2=Fucosyltransferase 2; FUT3=Fucosyltransferase 3; 3'-FL=3'-fucosyllactose; 2'-FL=2'-fucosyllactose; LNDFH I=Lacto-N-difucohexaose I.

Next, we performed unsupervised analysis of the NMR spectra to explore patterns representing underlying metabolic phenotypes on all samples. Although separation among the different HMO phenotypes was observed (Figure 6.3A-B), a subset showed a distinct metabolic signature characterized by a substantially lower lactose concentration than other samples (Figure 6.3B). We identified 45 (8.7%) samples with atypically low lactose concentration (Figure 6.3C) below a threshold of 128 mM (4.4 g/dL). We then investigated if the Se/Le status, could affect the concentration of lactose (Figure 6.3D). We show reduced concentration of lactose with presence of FUT2 and/or FUT3 however this did not account for the strikingly lower levels of lactose in the low-lactose subset. We also did not observe any statistically significant correlation between lactose concentrations and time postpartum, within the 5-10 week window for collection (Figure 6.3E).

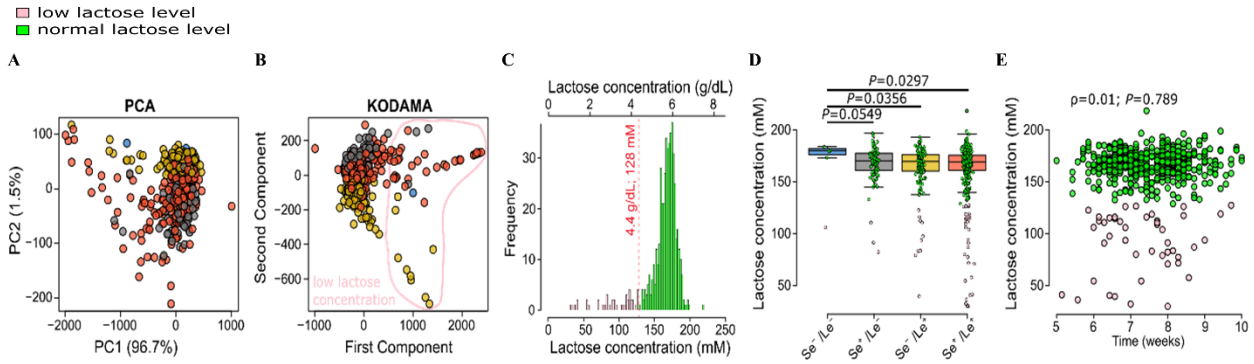

Figure 6. 3: Metabolic profiles of human breast milk (HBM) cluster according to lactose levels and HMO phenotype. (A) Principal components analysis (PCA) and, (B) unsupervised Knowledge Discovery by Accuracy Maximization (KODAMA) of HBM metabolic profiles. Major variance in the dataset, which was driven mainly by the lactose concentration, was explained by the first component of PCA (PC1). KODAMA shows separation of samples based on lactose concentration. (C) Distribution of lactose concentration and classification into low (pink) and normal (green) lactose levels. (D) Lactose concentration within the four different HMO phenotypes. P value is calculated by Wilcoxon rank sum test on the normal lactose level subset. (E) Lactose concentration over time postpartum. Spearman's coefficients (ρ) and P values are calculated on the normal lactose level subset.

6.3.3 Low lactose level associates with reduced duration of exclusive breastfeeding, lower infant growth and metabolites from mixed acid fermentation.

Since lactose is the major carbohydrate in HBM and plays a key role as a source of energy for the infant (46, 47), we explored the relationship between reduced lactose concentration and breastfeeding and infant outcomes. Strikingly, the median duration of exclusive breastfeeding was half as long (27.6 vs. 55.2 days, $P=2.61x10^{-4}$) in mothers with low (n=45) vs. normal (n=474) lactose concentrations (Figure 6.4 and Appendix 3, Table 2).

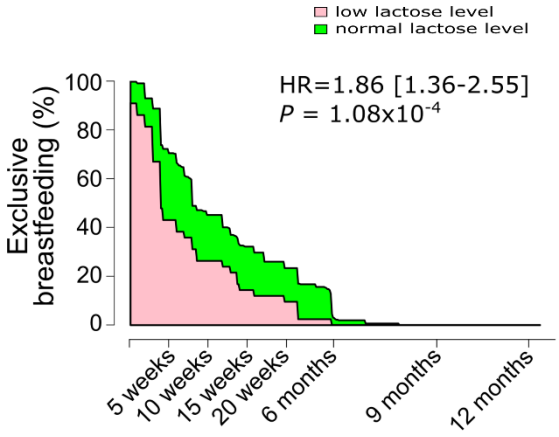

Figure 6. 4: Low lactose level in human breast milk (HBM) associates with reduced duration of exclusive breastfeeding. Kaplan-Meier plot shows duration of exclusive breastfeeding as assessed at the different clinic visits (Wald test was used to detect significance). HR: Hazard ratio. Low lactose samples are coloured pink and normal lactose samples green.

The proportion of calories from carbohydrate in HBM has been reported to positively correlate with infant weight, BMI and adiposity gains (48). Here, we found that infants breastfed by mothers with low HBM lactose had poorer anthropometric measures of growth, including lower z-scores for body length, body weight, and BMI; the differences in weight and BMI z-scores disappeared after the end of the exclusive breastfeeding period (Figure 6.5, Appendix 3, Table 3). The effect of low HBM lactose on infant length lasted longer (until 6 months) than the effect on weight, presumably since weight is a measure of acute nutritional status whilst length reflects longer-term effects.

We compared socio-demographic and psychosocial features between the groups of women whose HBM had normal and low levels of lactose (Appendix 3, Table 2). Low lactose level was more common amongst younger women ($P=1.21x10^{-2}$, Wilcoxon rank sum test). Daily consumption of organ meat, such as liver, heart, kidney, and tripe was less frequent ($P=2.01x10^{-2}$, Wilcoxon rank sum test), while daily consumption of fruit juice ($P= 2.46x10^{-2}$, Wilcoxon rank sum test) and cold drink ($P= 2.79x10^{-2}$, Wilcoxon rank sum test) were more frequent amongst women with low HBM lactose. Mothers with low HBM lactose were more likely to score above the threshold on Beck's Depression Inventory ($P=1.20x10^{-2}$, Fisher's exact test). Whilst we have few data on sleep or feeding patterns in the two groups, it would be interesting to explore the relationship between these patterns, low lactose HBM and maternal depression.

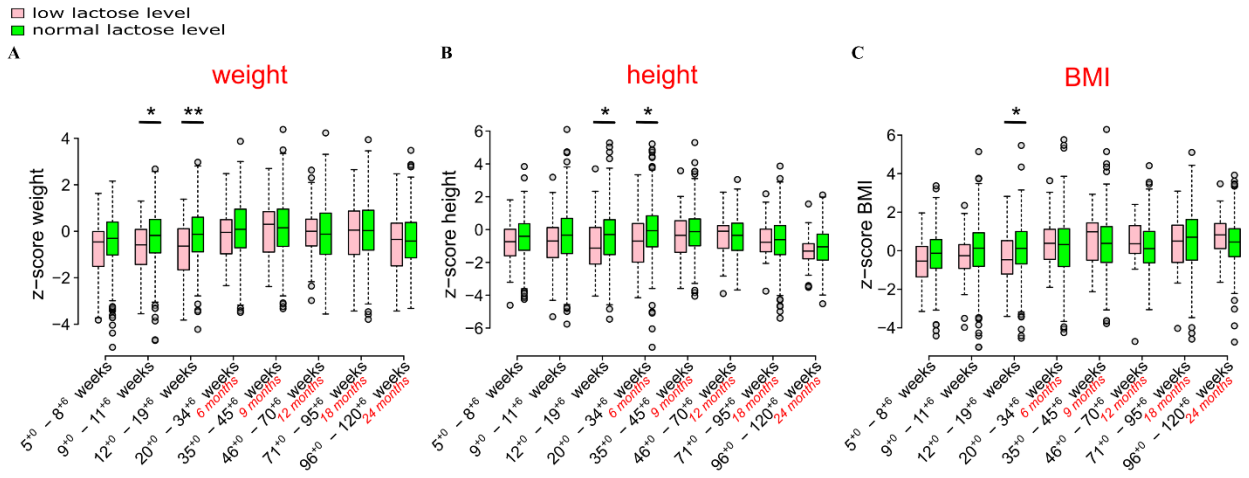

Figure 6. 5: Low lactose level in human breast milk (HBM) associates with lower infant growth. (A) Infant weight z-scores, (B) Infant height z-scores, and (C) Infant BMI z-scores at different study visits based on the level of lactose in the HBM. Low lactose samples are coloured pink and normal lactose samples green.

Next, we quantified 50 metabolites in all HBM samples (Appendix 3, Table 6) and we noted that the metabolic profile of samples with low lactose was very different from samples from the rest of the cohort (Figure 6.6A; Appendix 3, Table 4). Low lactose HBM samples showed evidence of mixed acid fermentation of lactose, with accumulation of pyruvate, lactate, acetate and formate, and a substantial reduction in all energetic

metabolites in the Krebs cycle (Figure 6.6B). Mixed acid fermentation is characteristic of microbial metabolism. Lactose is a major substrate for some micro-organisms including lactic acid bacteria which naturally occur in breast milk (49). There was also an increased concentration of uracil and its glycosylated form, uridine, in samples with low lactose (Figure 6.6B). Uridine is a precursor of uridine diphosphate (UDP) that plays a key role in the Leloir pathway to convert galactose (lactose is a disaccharide comprising galactose and glucose) into glucose.

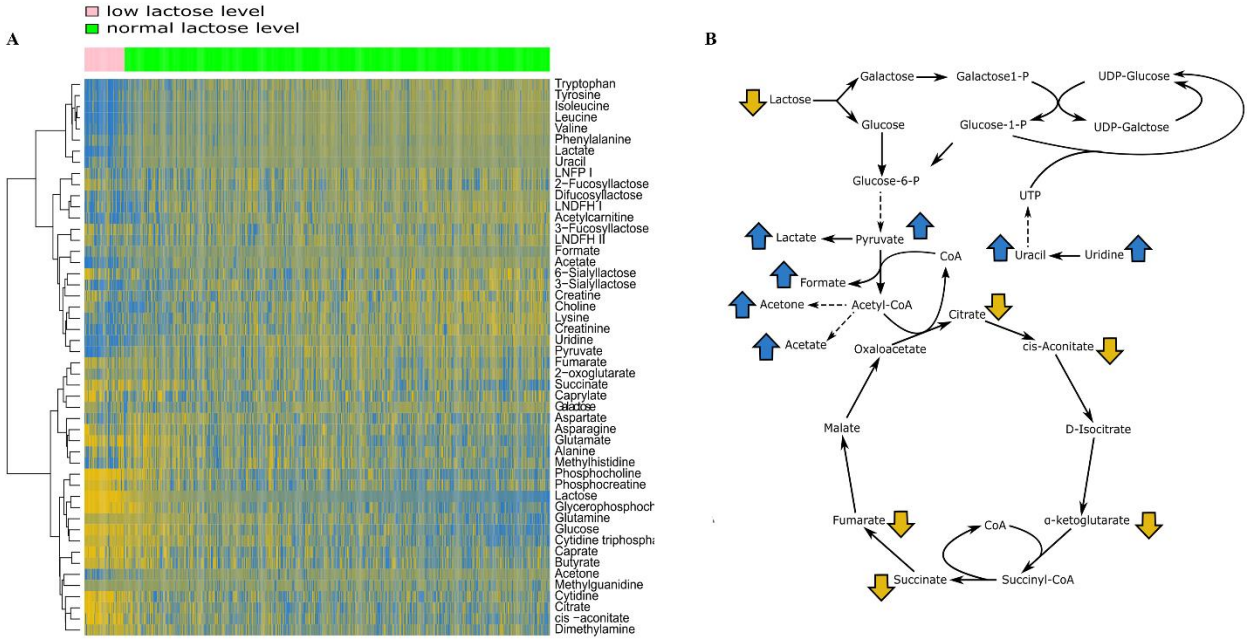

Figure 6. 6: Low lactose level in human breast milk (HBM) associates with metabolites from mixed acid fermentation. (A) Heatmap of metabolite concentrations in HBM samples. The samples were ordered based on the lactose concentration and metabolites using a hierarchical clustering algorithm. LNDFH II=Lacto-N-difucohexaose II; LNDFH I=Lacto-N-difucohexaose I; LDFT= Lactodifucotetraose; LNFP III= Lacto-N-fucopentaose III. (B) Schematic representation of metabolic changes in HBM samples with low lactose levels. Low lactose samples are coloured pink and normal lactose samples green.

6.3.4 Human breast milk (HBM) samples with low lactose level are associated with distinct bacterial profiles.

To evaluate the hypothesis that the depletion of lactose could be caused by fermentation associated with bacterial dysbiosis, we performed 16S rRNA gene amplicon sequencing (bacterial community analysis). In Figure 6.7A, we show bacterial profiles in HBM samples. We noted a strong association between bacterial profiles and lactose concentration. Strikingly higher abundance of *Staphylococcus* (order: Bacillales) and a lower abundance of *Streptococcus* and *Lactobacillus* (order: Lactobacillales), traditionally identified as using lactose as a substrate, was observed among low-lactose HBM samples. In addition, *Rothia*, *Gemella* and *Veillonella* which are members of the oral flora were also significantly lower among low-lactose HBM samples (Figure 6.7B, Appendix 3, Table 5). *Staphylococcus* is able to utilize lactose as energy source, with

particular animal-associated strains showing increased efficiency of lactose fermentation (50). We also observed a significantly higher absolute abundance of bacterial DNA in HBM with low lactose levels, as measured by quantitative polymerase chain reaction (PCR) targeting the 16S rRNA gene (Figure 6.7C).

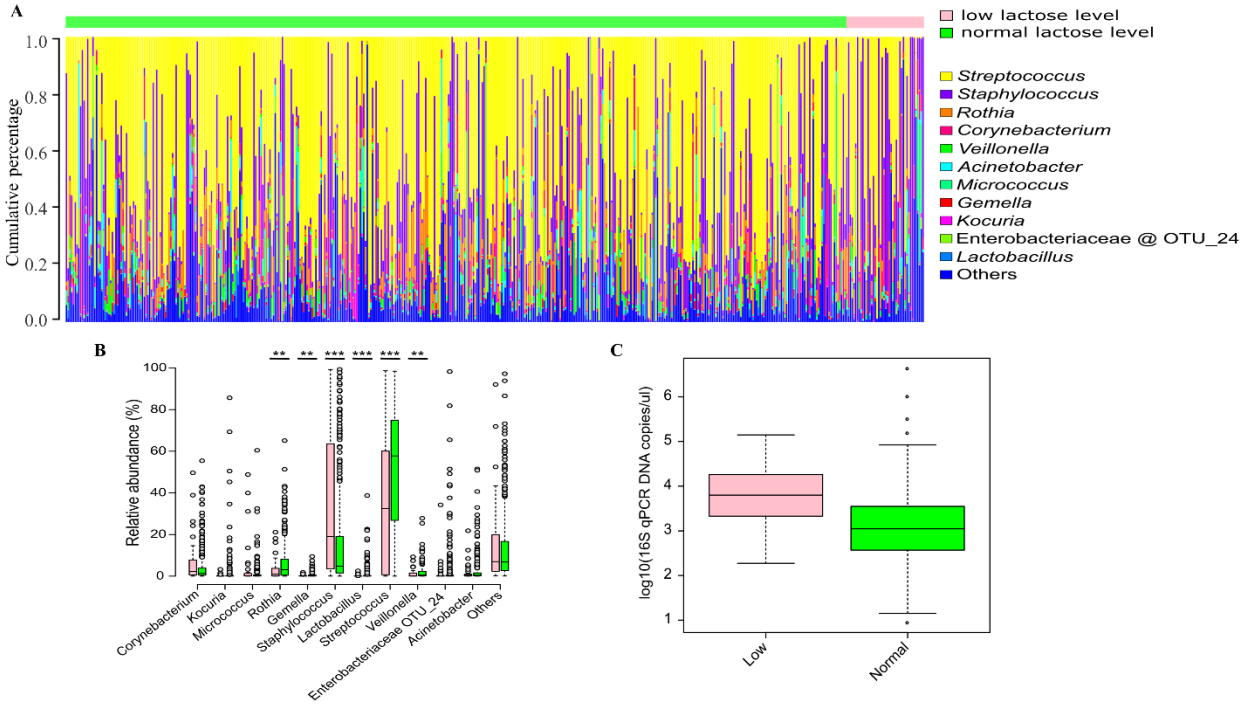

Figure 6. 7: Low lactose samples are associated with distinct bacterial profiles. (A) Relative abundances of HBM bacterial genera ordered based on the lactose concentration. Genera with a low relative abundance in a given sample are included as "Others". (B) Boxplot showing differences in relative abundances of the most abundant bacterial genera between samples with low and normal lactose level, ** P<0.01, *** P<0.001. The line inside the box plot represents the median, while the box signifies the 75% (upper) and 25% (lower) quartile showing the distribution of 50% of the samples. P is calculated by Wilcoxon rank sum test. (C) 16S qPCR DNA copies/ul values of samples with low vs. normal lactose levels. The line inside the box plot represents the median, while the box signifies the 75% (upper) and 25% (lower) quartile showing the distribution of 50% of the samples. P is calculated by Wilcoxon rank sum test.

Unsupervised analysis of HBM bacterial profiles (Figure 6.8A) revealed three bacterial signatures, distinguished by the relative abundances of *Staphylococcus* spp. and/or *Streptococcus* spp (Figure 6.8B). We observed a higher percentage of samples with low lactose in signature 2 dominated by *Staphylococcus* spp. Mastitis, an infection of the breast tissue in breastfeeding women, is most commonly caused by staphylococcal infection (51) and has previously been associated with relatively lower levels of lactose in HBM (52, 53). The women in our cohort did not report symptoms of mastitis, however it is possible that sub-clinical mastitis may have been present. Whilst the phenomenon of subclinical mastitis has been reported in animals in the dairy industry (54, 55), it is not widely recognized as a distinct condition in humans.

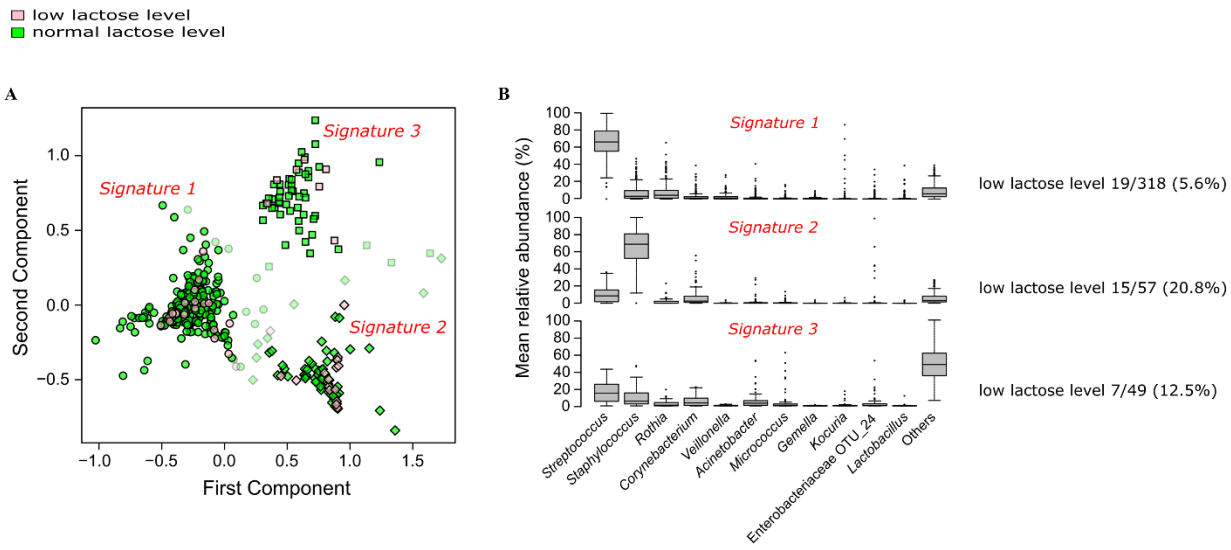

Figure 6. 8: Low lactose samples are associated with distinct bacterial signatures. (A) Unsupervised KODAMA of the HBM bacterial profile data showing three different bacterial signatures. (B) Boxplots of the relative abundance of major bacterial genera in the three different signatures and proportion of samples with low lactose. Low lactose samples are coloured pink and normal lactose samples green throughout.

6.4 CONCLUSION

In summary, we describe here a subset of breastfeeding women whose HBM had very low levels of lactose. Low lactose was associated with reduced duration of exclusive breastfeeding and poorer infant growth during the period of exclusive breastfeeding. Low lactose concentration was associated with HBM bacterial dysbiosis (evidence of mixed acid fermentation of lactose by an HBM bacteriome dominated by staphylococci). Our study has several limitations.

Despite the large sample size, the population studied comes from a single semi-rural geographical setting. This was a cross-sectional study with only one sampling time point; we were not able to assess whether this phenotype is persistent. Further work is required to determine whether this finding is generalizable to other populations or stages of lactation. If these findings are replicated in other populations, they may have relevance for the health of mother and child populations, and consideration should be given to diagnostic and therapeutic studies.

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CHAPTER 7

GENERAL CONCLUSIONS

HBM is a unique physiological fluid adapted for the growth and wellbeing of an infant. Its composition is complex; this thesis focuses on two components: the bacteriome and metabolome. The HBM bacteriome is diverse but understudied. We used culture independent techniques, specifically, next generation sequencing to explore its composition and determinants. The metabolome also has an important role in infant health and is increasingly studied for its nutritive and bioactive molecules; here we studied the metabolome in the context of the associated microbiome.

In the first two chapters, we reviewed the scientific literature on the constituents of the HBM bacteriome and metabolome, the role they play in infant health and well-being, and their determinants including maternal, infant and environmental factors. We also identified existing gaps in HBM research – and these include limited studies on HBM bacteriome and metabolome and their influencing factors in low socio-economic regions, particularly in African populations. Furthermore, there are few integrative studies exploring the interplay between the HBM bacteriome and the metabolome, and their collective role in infant health and growth.

The first part of the original research towards this thesis highlighted the importance of optimisation of laboratory technical procedures in HBM bacteriome research, including DNA extraction as well as milk skimming on HBM bacterial profiles. Illumina MiSeq sequencing targeting the V4 region of the 16S rRNA gene was used to assess the bacterial profiles using ten healthy donor breast milk samples. Although optimization of DNA extraction kits has been conducted on other biological samples, to the best of our knowledge, this is the first of such studies to be published on HBM samples. We showed that DNA extraction kits, but not milk skimming, impacted significantly on both DNA yield and purity, the recovery of specific bacterial genera, and the efficiency of DNA extraction from bacterial communities present in a commercial mock microbial community standard spiked into HBM. Although the extraction kits all gave similar bacterial diversity measures, the relative abundances of bacteria obtained with each kit differed. An interesting observation was the influence of benzonase pre-treatment on DNA recovery, a step included in one of the kits used in this optimisation step. Benzonase treatment led to low DNA yield, and biased representation of bacteria in the mock community. Since this kit is routinely recommended for microbiome studies, optimization of extraction kits and methods is important as this influences the downstream analysis of next generation sequencing experiments.

The result of this optimisation was that ZR Fungal/Bacterial DNA MiniPrep™ was chosen for further DNA extraction from HBM samples. This was based on the higher DNA yield obtained, best DNA purity measures, and its ability to most accurately represent the bacteria in the mock community.

In the next research chapter of this thesis, the bacteriome of HBM in a cohort of lactating women living in South Africa was comprehensively described. By analysing the V4 region of the 16S rRNA gene of HBM bacterial communities, we confirmed that a diverse bacteriome exists. We identified a core bacteriome that is

shared among 80% of samples. Three bacterial cluster groups (biotypes) were identified based on the relative abundances of *Staphylococcus* and/or *Streptococcus*. Inter-individual variability was observed in the bacterial profiles of samples. A major strength of this study is the detailed metadata collected. A robust multivariate statistical analysis showed that study site (a proxy for ethnicity) was an important factor associated with the composition of the HBM microbiome. While the HBM samples from Mbekweni had significantly higher relative abundance of the genus *Streptococcus* (which has been associated with health), HBM samples from TC Newman had significantly higher relative abundances of the genera *Staphylococcus, Acinetobacter, and Escherichia* Maternal age influenced the diversity of HBM bacterial profiles, with older mothers having a higher Shannon diversity index. Though limited by the cross-sectional design of this study, a longitudinal study would have afforded us the opportunity to study the determinants of the HBM bacteriome (i.e. antibiotics use, diet and maternal stress) over time.

Lastly, correlations between bacterial abundances were observed within the HBM bacteriome, notably between bacteria that are members of the skin microbiota, and, separately, those that are members of the oral microbiota. Although not within the scope of this research, research has shown that HBM bacteria originates, among others, from the oral microbiome of the infant due to a retrograde flow of milk during suckling, and from the surrounding breast skin and areola which is open to the external environment. It is therefore not surprising to see interactions between bacteria based on the assumed origin. Being limited by the targeted 16S rRNA sequence analysis approach, we could not assess the functional capacity of the bacterial communities within the HBM. Future work will involve the use of shotgun metagenomic sequencing to comprehensively sample all genes of all bacterial organisms present to determine species-level identity and functional capacity and relate this to infant gut health.

The final stage of this thesis investigated the metabolome of HBM. The nutritive and non-nutritive bio-active metabolites in HBM are considered extremely important in the first six months of life. We were able to detect a total of 49 metabolites from HBM samples included in this study, with nuclear magnetic resonance spectroscopy. More importantly, a subset of lactating women with unusually low lactose concentration in their HBM sample was detected in the cohort. This subset of women exclusively breastfed for shorter than the rest of the cohort, and their infants demonstrated lower growth during the period of exclusive breastfeeding. This subset of women also had a higher prevalence of depression. Metabolites characteristic of mixed acid fermentation were also observed in the HBM samples of this subset of women, and this was associated with a dysbiotic, staphylococcal-predominant microbiota. While the cross-sectional study design employed in this study and the peri-urban settings from which participants are recruited limits the generalizability of these results, the use of NMR spectroscopy provides new insight to detecting variability in HBM metabolites.

In summary, this thesis presents a detailed description of the bacteriome, and metabolome in HBM samples using 16S rRNA gene amplicon sequencing and nuclear magnetic resonance spectroscopy respectively. We identified factors that influence the HBM composition and showed that HBM bacteriome and metabolome

modulates the growth of an infant and breastfeeding outcomes. The results presented in this thesis clearly highlight the need for further HBM research in an African setting.

In addition to future work highlighted in each research chapter, future work will include:

i. Characterizing the HBM lipidome in this cohort of lactating South African mothers enrolled in the Drakenstein Child Health Study.
ii. Determining maternal, infant and environmental factors that influences the HBM lipidome in this cohort.
iii. Investigating the association between HBM lipidome and infant growth in the first year of life and determining the association (if any) between these three components of HBM.

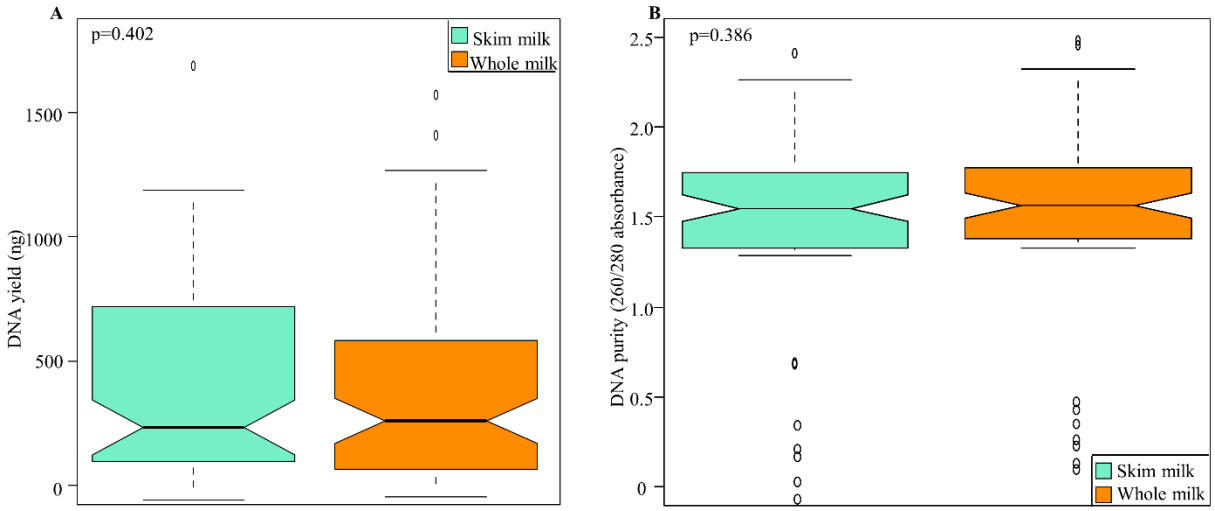
APPENDIX 1: Chapter 4 supplementary tables and figures

Appendix 1, Table 1: Zymobiomics™ microbial community standard composition as per manufacturer's specifications.

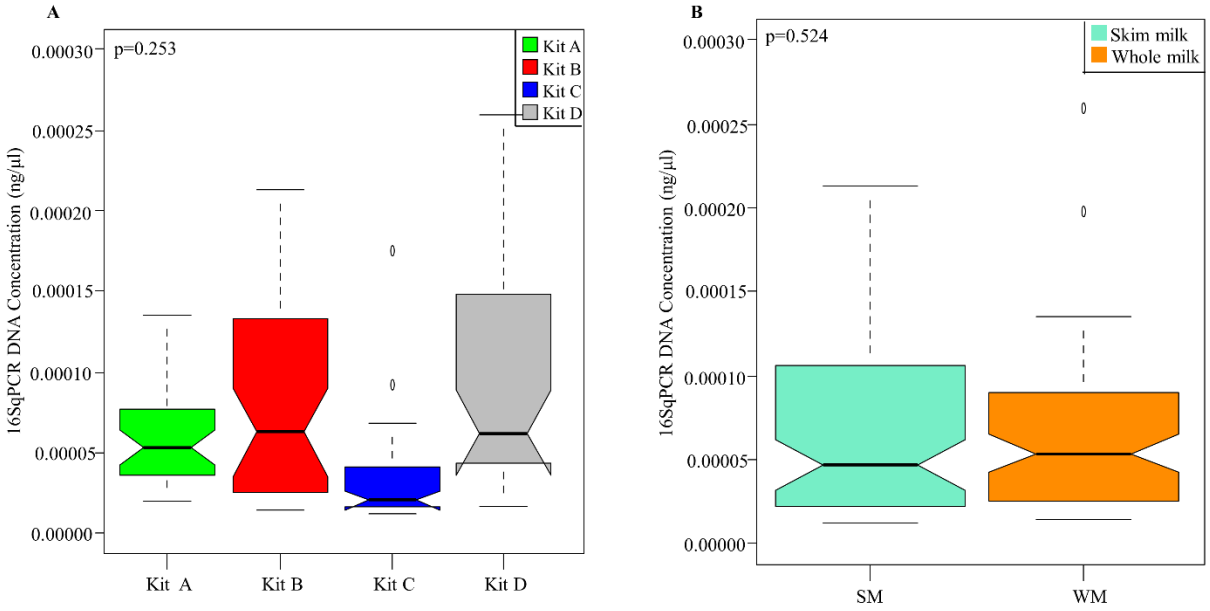
Bacteria species	gDNA abundance (%)	mOTU abundance (%)	Gram stain
Pseudomonas spp.	12%	4.6	-
Enterobacteriaceae 1	12%	11.3	-
Enterobacteriaceae 2	12%	10.0	-
Listeria spp.	12%	15.9	+
Staphylococcus spp.	12%	13.3	+
Lactobacillus spp.	12%	18.8	+
Enterococcus spp.	12%	10.4	+
Bacillus spp.	12%	15.7	+

Appendix 1, Table 2: Primers, probes and cycling conditions

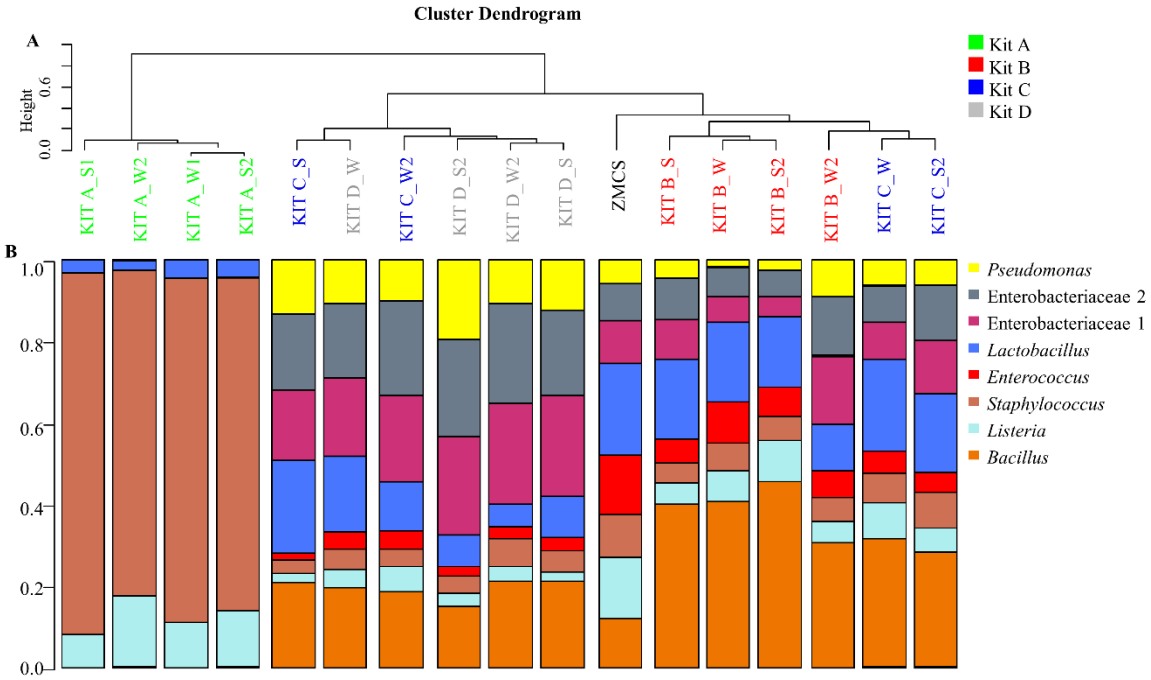
		Forward primer	Reverse primer	Probe	PCR cycling conditions
A	qPCR	16S-F1(5′-CGA AAG CGT GGG GAG CAA A -3′)	16S-R1(5′-GTT CGT ACT CCC CAG GCG G-3′)	16S-P1 (FAM-ATT AGA TAC CCT GGT AGT CCA –MGB	45 cycles 50 ºC 95 ºC 95 ºC 60 °C 72 °C 2min 10min 15s 60s 1s
B	Short PCR	(515Fshort) GTGCCAGCHGCYGCGGT	(806Rshort) GGACTACNNGGGTNTCTAAT	-	10 cycles 95 ºC 95 ºC 50 °C 72 °C 72 °C 4 °C 3min 30s 30s 1s 5min ∞
C	Long PCR	(515Flong) GTGCCAGCHGCYGCGGT	(806Rlong_barcoded) GGACTACNNGGGTNTCTAAT	-	30 cycles 95 ºC 95 ºC 50 °C 72 °C 72 °C 4 °C 3min 30s 30s 1s 5min ∞



Appendix 1, Figure 1: DNA yield and purity based on skim milk and whole milk. Notched box plots showing (A) the DNA yield and (B) the DNA purity (260/280 absorbance) obtained from skim and whole milk. The notched box signifies the 75% (upper) and 25% (lower) quartile showing the distribution of 50% of the samples. The line inside the box plot represents the median, and the notch the 95% confidence interval for the median. The whiskers (top and bottom) represent the maximum and minimum values. Outliers, which are beyond 1.5 times the interquartile range above the maximum value and below the minimum value, are shown with open circles.



Appendix 1, Figure 2: 16SqPCR DNA concentration based on kit and milk type (skim milk and whole milk). Notched box plots showing the 16SqPCR DNA concentration obtained by using (A) the kits, and (B) skim and whole milk. The notched box signifies the 75% (upper) and 25% (lower) quartile showing the distribution of 50% of the samples. The line inside the box plot represents the median, and the notch the 95% confidence interval for the median. The whiskers (top and bottom) represent the maximum and minimum values. Outliers, which are beyond 1.5 times the interquartile range above the maximum value and below the minimum value, are shown with open circles. SM=skim milk, WM=whole milk.



Appendix 1, Figure 3: Complete linkage hierarchical clustering of human breast milk spiked with the microbial mock community standard. (A) Dendogram of bacterial profiles present in the HBM sample spiked with ZMCS processed as skim and whole milk using four DNA extraction kits. (B) Relative abundances of ZMCS bacterial composition in the HBM sample spiked with ZMCS. Each column represents milk type (skim or whole) extracted by one of the four kits. W: whole milk, S: skim milk; S2 and W2: replicates of S and W respectively. ZMCS: Zymobiomics Microbial Community Standard reference ("theoretical" bacterial profile) specified by the manufacturer.

Appendix 1, Table 3: Bacterial 16S DNA concentration in the spiked and un-spiked human breast milk sample.

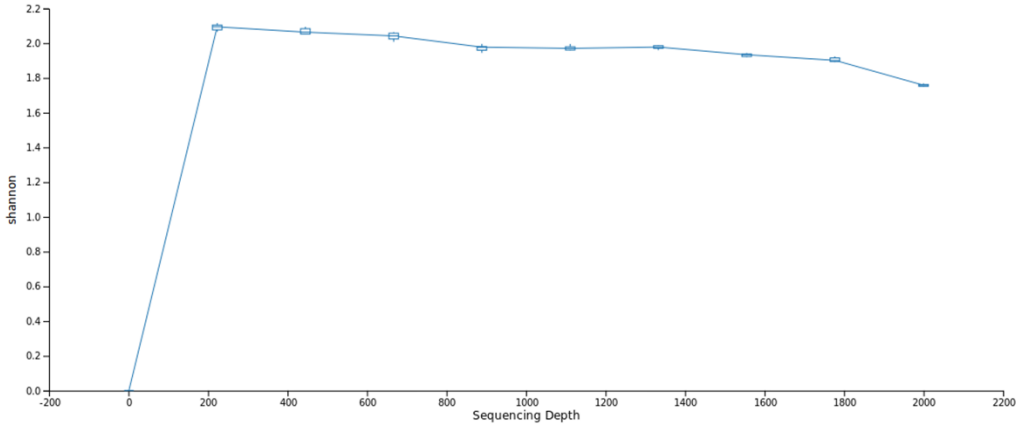
Sample_ID	Spike/Unspiked_Kit	Donor sample	Template DNA 16SqPCR concentration (ng/ul)	Median 16S qPCR DNA concentration	%16S DNA in HBM sample
BM_AO_KITdnam_extractioncontrolspike_1_kitcomparison_P3_A06	Spiked_Kit A	1	0.251124139	0.195487886	99.975%
BM_AO_KITdnam_extractioncontrolspike_1_kitcomparison_P3_A07	Spiked_Kit A	1	0.175701367		
BM_AO_KITdnam_extractioncontrolspike_1_kitcomparison_P3_B06	Spiked_Kit A	1	0.215274404		
BM_AO_KITdnam_extractioncontrolspike_1_kitcomparison_P3_B07	Spiked_Kit A	1	0.047838204		
BM_AO_KITdnam_skim_1_kitcomparison_P3_C01	Unspiked_Kit A	1	0.000047258	0.000047105	0.024%
BM_AO_KITdnam_skim_1_kitcomparison_P3_C08	Unspiked_Kit A	1	0.000046952		
BM_AO_KITdnam_whole_1_kitcomparison_P3_A01	Unspiked_Kit A	1	0.000068726		
BM_AO_KITdnam_whole_1_kitcomparison_P3_A08	Unspiked_Kit A	1	0.000045226		
BM_AO_KITqias_extractioncontrol_1_kitcomparison_P4_A06	Spiked_Kit C	1	0.332685221	0.151318187	99.983%
BM_AO_KITqias_extractioncontrol_1_kitcomparison_P4_A07	Spiked_Kit C	1	0.293789111		
BM_AO_KITqias_extractioncontrol_1_kitcomparison_P4_B06	Spiked_Kit C	1	0.002849745		
BM_AO_KITqias_extractioncontrol_1_kitcomparison_P4_B07	Spiked_Kit C	1	0.008847263		
BM_AO_KITqias_skim_1_kitcomparison_P4_C01	Unspiked_Kit C	1	0.000060109	0.000025238	0.016%
BM_AO_KITqias_skim_1_kitcomparison_P4_C08	Unspiked_Kit C	1	0.000024687		
BM_AO_KITqias_whole_1_kitcomparison_P4_A01	Unspiked_Kit C	1	0.000025790		
BM_AO_KITqias_whole_1_kitcomparison_P4_A08	Unspiked_Kit C	1	0.000020727		
BM_AO_KITzymob_extractioncontrol_1_kitcomparison_P4_G06	Spiked_Kit D	1	1.214408365	0.843423774	99.989%
BM_AO_KITzymob_extractioncontrol_1_kitcomparison_P4_G07	Spiked_Kit D	1	0.397619204		
BM_AO_KITzymob_extractioncontrol_1_kitcomparison_P4_H06	Spiked_Kit D	1	0.792653652		
BM_AO_KITzymob_extractioncontrol_1_kitcomparison_P4_H07	Spiked_Kit D	1	0.894193897		
BM_AO_KITzymob_skim_1_kitcomparison_P4_G01	Unspiked_Kit D	1	0.000066957	0.000087592	0.01%
BM_AO_KITzymob_skim_1_kitcomparison_P4_G08	Unspiked_Kit D	1	0.000108227		
BM_AO_KITzymob_whole_1_kitcomparison_P4_E01	Unspiked_Kit D	1	0.000059373		
BM_AO_KITzymob_whole_1_kitcomparison_P4_E08	Unspiked_Kit D	1	0.000198393		
BM_AO_KITzymo_extractioncontrol_1_kitcomparison_P3_G06	Spiked_Kit B	1	0.703367972	0.898759654	99.984%
BM_AO_KITzymo_extractioncontrol_1_kitcomparison_P3_G07	Spiked_Kit B	1	1.094151336		
BM_AO_KITzymo_extractioncontrol_1_kitcomparison_P3_H06	Spiked_Kit B	1	0.654916126		
BM_AO_KITzymo_extractioncontrol_1_kitcomparison_P3_H07	Spiked_Kit B	1	1.123935714		
BM_AO_KITzymo_skim_1_kitcomparison_P3_G01	Unspiked_Kit B	1	0.000194474	0.000141692	0.015%

BM_AO_KITzymo_skim_1_kitcomparison_P3_G08	Unspiked_Kit B	1	0.000211923
BM_AO_KITzymo_whole_1_kitcomparison_P3_E01	Unspiked_Kit B	1	0.000025031
BM_AO_KITzymo_whole_1_kitcomparison_P3_E08	Unspiked_Kit B	1	0.000088910

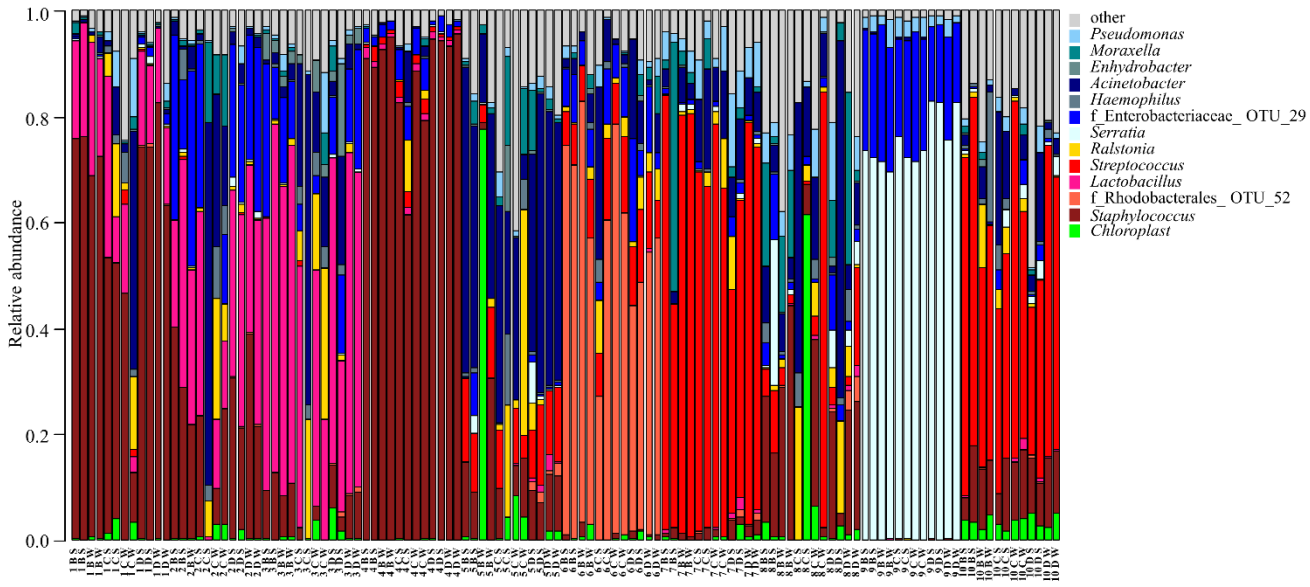
ZMCS: Zymobiomics Microbial Community Standard; HBM: Human breast milk

Appendix 1, Table 4: Beta diversity (Bray-Curtis dissimilarity index) measuring dissimilarity between Zymobiomics microbial community standard (ZMCS) and the milk type, for each of the kits.

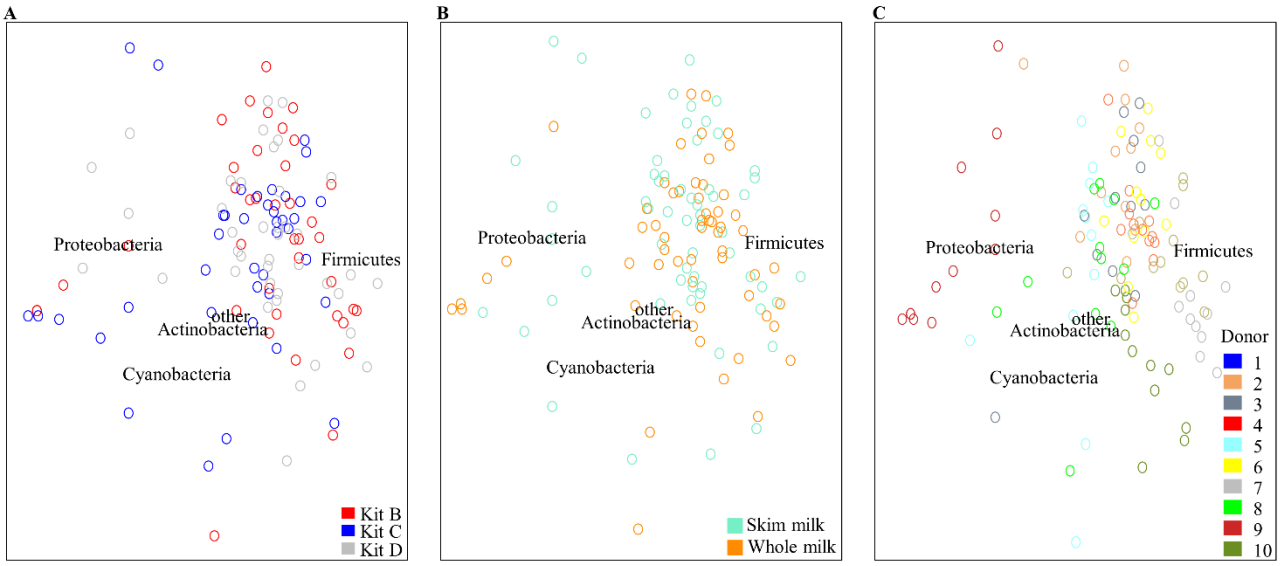
	Milk type	
Kits	Skim milk	Whole milk
Kit A vs. ZMCS	0.29	0.25
Kit B vs. ZMCS	0.06	0.16
Kit C vs. ZMCS	0.16	0.16
Kit D vs. ZMCS	0.11	0.20



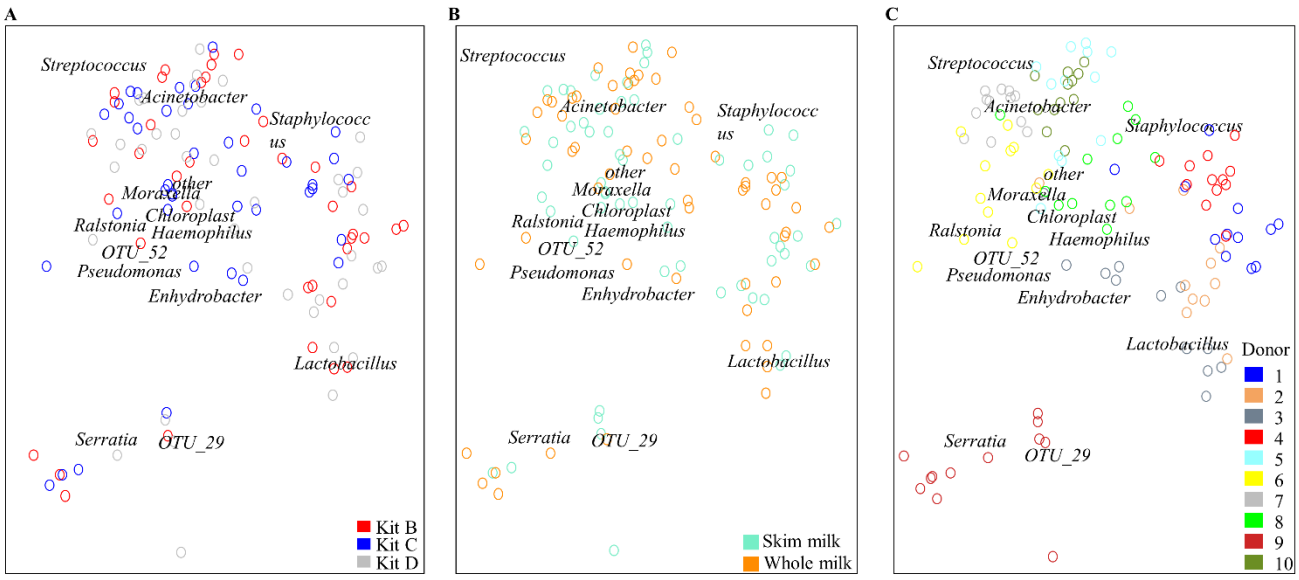
Appendix 1, Figure 4: Alpha diversity rarefaction curve. A line graph relating the sequencing depth to Shannon diversity index of sequenced un-spiked breast milk samples at a sub-sampling depth of 100.



Appendix 1, Figure 5: Relative abundances of bacterial composition in un-spiked human breast milk samples at genus level. Each bar represents a sample and each coloured box, a bacterium. Genera less than 0.5% abundance in a given sample are included in the "other" at the top of the graph (grey boxes).



Appendix 1, Figure 6: Log ratio biplot human of human breast milk bacterial abundances at the phylum level. Samples are coloured according to (a) extraction kit used, and (b) whole and skim milk (c) donors. Points (circles) that cluster together are similar in bacterial composition and abundance.



Appendix 1, Figure 7: Log ratio biplot human of human breast milk bacterial abundances at the genus level. Samples are coloured according to (a) extraction kit used, and (b) whole and skim milk (c) donors. Points (circles) that cluster together are similar in bacterial composition and abundance.

Appendix 1, Table 5: Differential abundances of statistically significant bacteria taxa in relation to extraction kits and milk type in un-spiked breast milk samples.

Taxonomy level	Kit B	Kit C	Kit D	p-value
KITS				
Proteobacteria	0.3436	0.4761	0.3772	0.005
Alphaproteobacteria	0.0135	0.0162	0.0093	0.001
Sphingomonadales	0.0058	0.0087	0.0039	<0.001
Rhodobacterales				
OTU_52	0.0712	0.0564	0.0561	0.035
Betaproteobacteria	0.0294	0.089	0.0271	<0.001
Burkholderiales	0.0244	0.0856	0.0242	<0.001
Burkholderiaceae	0.007	0.0758	0.0176	<0.001
Ralstonia	0.007	0.075	0.0175	<0.001
Gammaproteobacteria				
Pseudomonadales	0.1157	0.2086	0.1607	<0.001
Pseudomonadaceae	0.0167	0.0259	0.024	0.016
Pseudomonas	0.0165	0.0259	0.024	0.016
Pasteurellales	0.1109	0.2205	0.0045	<0.001
Pasteurellaceae	0.011	0.2205	0.0045	<0.001
Haemophilus	0.011	0.022	0.0045	<0.001
Moraxellaceae				
Acinetobacter	0.0606	0.1544	0.1026	<0.001
Firmicutes				
Bacillus				
Lactobacillales				

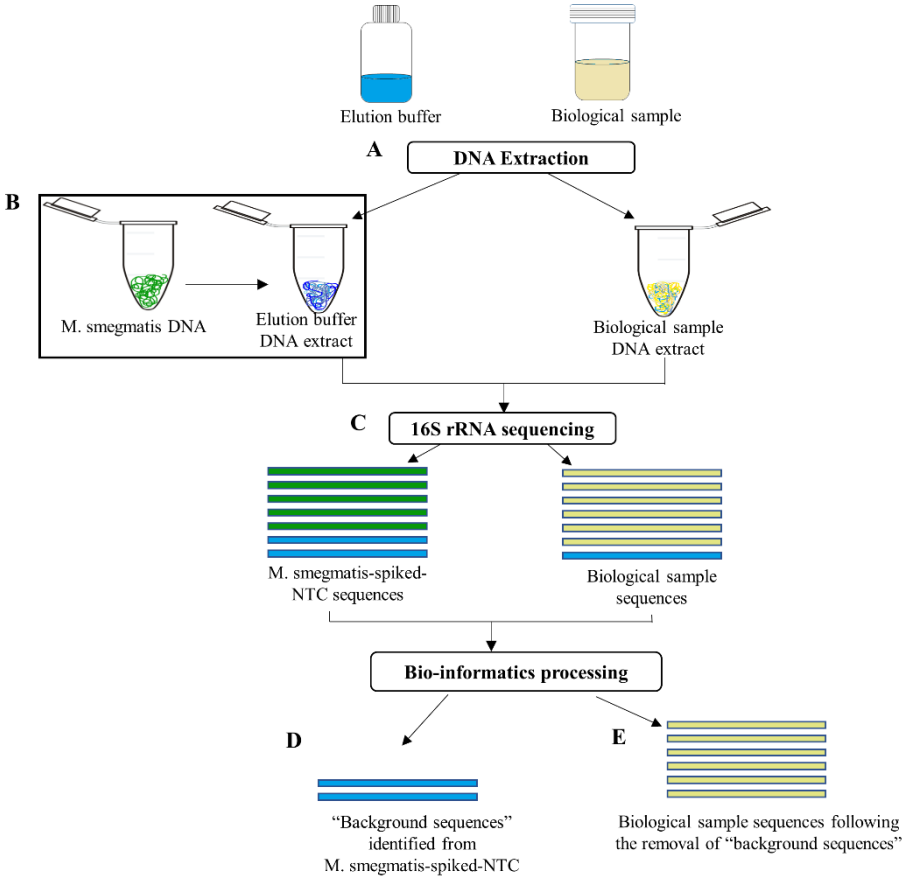
Lactobacillaceae	0.1084	0.0632	0.1088	<0.001
Lactobacillus	0.1084	0.0632	0.1088	<0.001
WM/SM				
	Skim milk	Whole milk		
Pseudomonadales	0.1948	0.1288	--	0.009
Moraxellaceae	0.1721	0.1068	--	0.004
Enhydrobacter	0.0078	0.0039	--	0.002
Acinetobacter	0.1353	0.0767	--	0.003

Mean values of relative abundance proportions for statistically significant bacteria at different taxonomy levels are shown. p-values are Benjamini-Hochberg corrected. p-values are represented as '***' 0.001; '**' 0.01; and '*' 0.05. WM and SM represents whole milk and skim milk respectively.

Appendix 1, Table 6: Reproducibility of extractions tested using multiple R-squared.

	Multiple R-square	
	Whole milk	Skim milk
Kit B	0.4908	0.7581
Kit C	0.7421	0.4751
Kit D	0.9483	0.7976

APPENDIX 2: Chapter 5 supplementary tables and figures



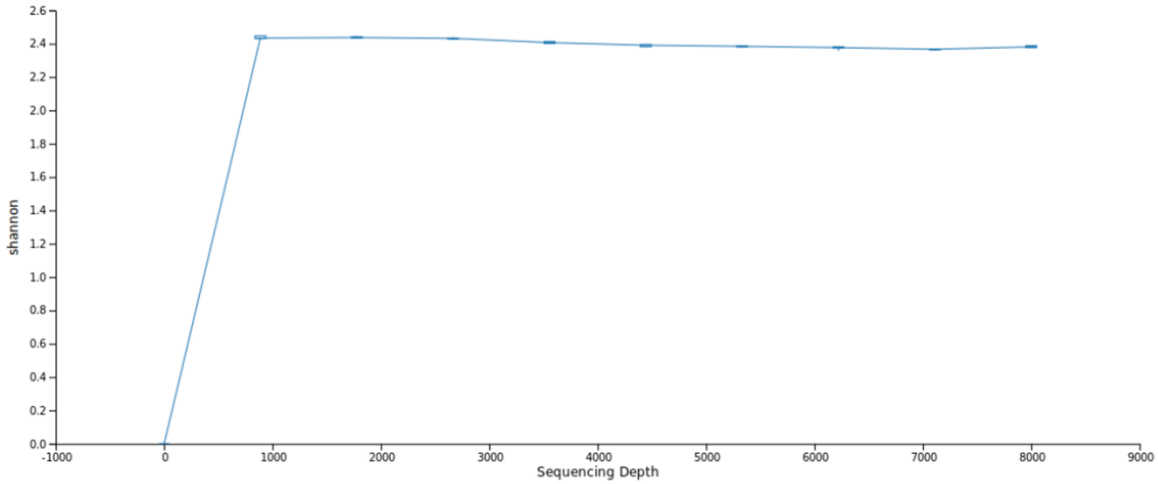
Appendix 2, Figure 1: In-silico de-contamination of biological human breast milk samples.

Appendix 2, Table 1: Demographic and clinical characteristics of mothers and their infants

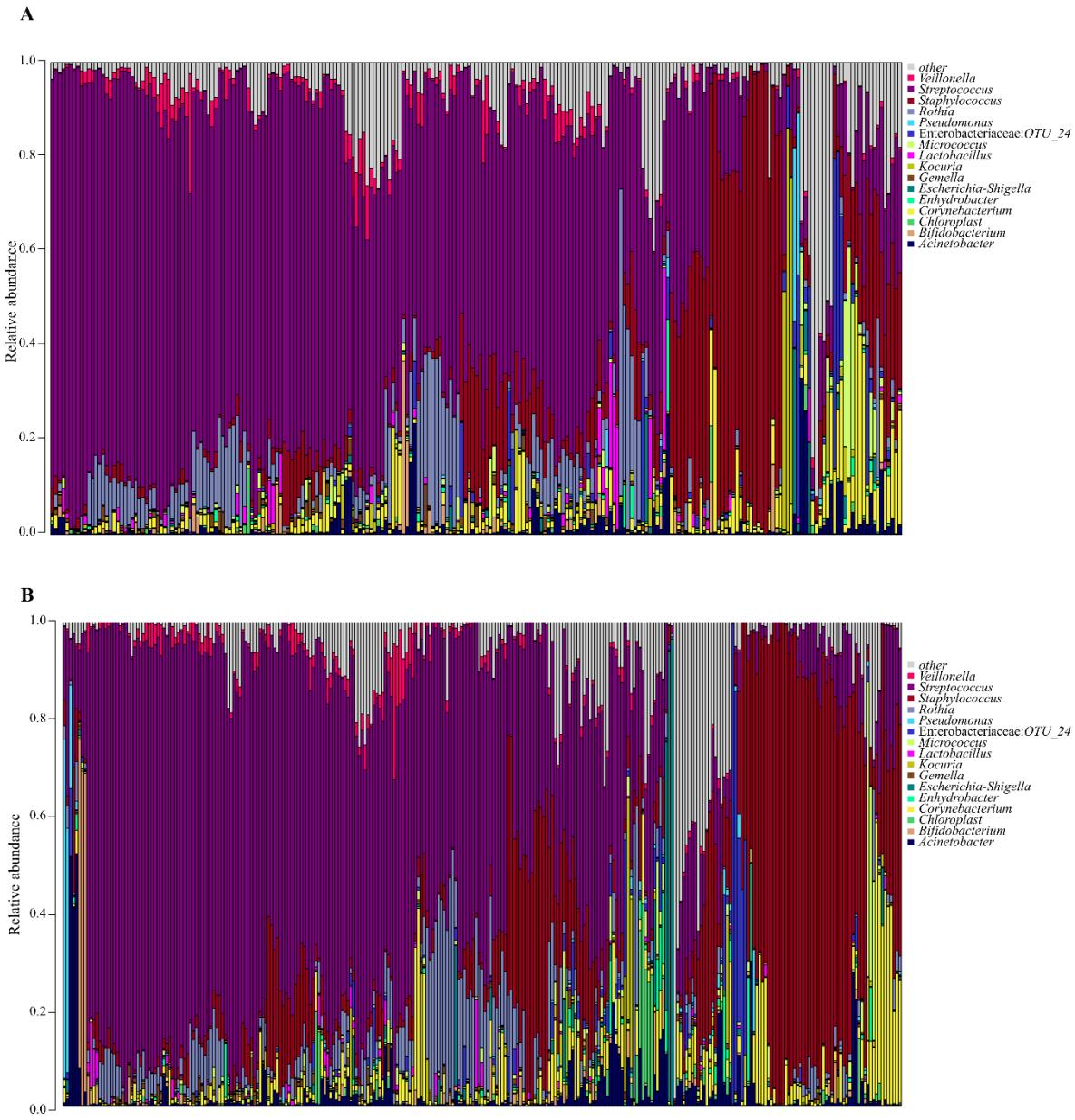
	Mbekweni	TC Newman	Both study sites
Maternal demographic and psychosocial characteristics			
Maternal age at enrolment (years), median [95%CI]	25.5 [18.5 39.5]	25.3 [18.7 39.6]	25.3 [18.6 39.6]
Maternal weight (kg), median [95%CI]	68.1 [48 110.6]	59.5 [42.7 95.3]	63.7 [43.7 102.3]
Maternal BMI, median [95%CI]	26.7 [19.1 41.1]	23.6 [17.6 37.7]	25 [17.9 40.6]
Lactation stage (weeks), median [range]	7.7 [5.3 19.5]	7.4 [2.4 18.7]	7.5 [2.4 19.5]
Ethnicity, n (%)			
Black African	244 (99.2%)	0 (0.0%)	244 (44.0%)
Mixed ancestry	2 (0.8%)	308 (100.0%)	310 (56.0%)
Maternal education, n (%)			

Primary	152 (61.8%)	191 (62.0%)	343 (61.9%)
Secondary	72 (29.3%)	102 (33.1%)	174 (31.4%)
Tertiary	22 (8.9%)	15 (4.9%)	37 (6.7%)
Mother employed, n(%)	56 (22.8%)	79 (25.6%)	134 (24.4%)
Household income, n (%)			
<ZAR1000/m	126 (51.2%)	99 (32.1%)	225 (40.6%)
>ZAR5000/m	17 (6.9%)	58 (18.8%)	75 (13.5%)
ZAR1000-5000/m	103 (41.9%)	151 (49.0%)	254 (45.8%)
HIV infected mothers, n(%)	46 (18.7%)	6 (1.9%)	52 (9.4%)
Maternal smoking status, n (%)			
Active smoker	30 (12.9%)	154 (51.9%)	184 (34.8%)
Non-smoker	81 (34.9%)	30 (10.1%)	111 (21.0%)
Passive smoker	121 (52.2%)	112 (38.0%)	234 (44.2%)
Infant feeding at 6-10 weeks, n (%)			
Exclusive breastfeeding	131 (54.8%)	155 (51.2%)	286 (52.8%)
Mixed breastfeeding	108 (45.2%)	148 (48.8%)	256 (47.2%)
Exclusive breastfeeding at 4 months, n (%)	66 (27.5%)	82 (27.0%)	148 (27.2%)
Mode of delivery, n (%)			
Elective C/S	18 (7.4%)	16 (5.2%)	34 (6.2%)
Emergency C/S	37 (15.2%)	34 (11.0%)	71 (12.9%)
Vaginal	189 (77.5%)	258 (83.8%)	447 (81.0%)
Maternal PTSD, n (%)			
No exposure	178 (83.6%)	243 (86.2%)	421 (85.1%)
Suspected PTSD	20 (9.4%)	16 (5.7%)	36 (7.3%)
Trauma exposed	15 (7.0%)	23 (8.2%)	38 (7.7%)
Maternal BDI Threshold, n (%)			
Above threshold	49 (22.5%)	77 (27.3%)	126 (25.2%)
Below threshold	169 (77.5%)	205 (72.7%)	374 (74.8%)
Maternal SRQ Threshold, n (%)			
Above threshold	41 (18.7%)	71 (25.2%)	112 (22.4%)
Below threshold	178 (81.3%)	211 (74.8%)	389 (77.6%)
Household size, median [95%CI]	4 [1 11]	5 [2 12]	5 [2 12]
Maternal alcohol score, median [95%CI]	0 [0 30.2]	0 [0 25.9]	0 [0 26]
Infant characteristics			
Infant weight for age z-score, median [95%CI]	-0.36 [-2.5 1.86]	-0.74 [-2.92 1.06	-0.6 [-2.81 1.62]
Infant height for age z-score, median [95%CI]	0.1 [-3.44 2.74]	-0.27 [-3.44 2.11]	0 [-3.4 2.6]
Infant gestational age, n (%)			
Preterm	55 (22.4%)	71 (23.1%)	126 (22.7%)
Term	184 (74.8%)	230 (74.7%)	414 (74.7%)
Very preterm	7 (2.8%)	7 (2.3%)	14 (2.5%)
Infant gender, n (%)			
Female	122 (49.6%)	143 (46.6%)	265 (47.8%)
Male	123 (50.0%)	165 (53.6%)	288 (52.0%)
Triplets	1 (0.4%)	0 (0.0%)	1 (0.2%)

BMI=Body Mass Index; HIV= Human Immunodeficiency Virus; EBF=Exclusive breastfeeding; PTSD= post-traumatic stress disorder; BDI= Beck Depression Inventory; SRQ= Self-Regulation Questionnaire; Smoking status was measured as Active smoker (cotinine levels ≥ 500); Passive smoker (cotinine levels 10 – 500); Non-smoker (cotinine levels ≤ 10). Term, Preterm and Very-preterm infants are infant with gestational age of >37 weeks, 32-37 weeks and 28-31.9 weeks respectively. C/S=caesarean section.



Appendix 2, Figure 2: Alpha rarefaction curve relating the sequencing depth to Shannon diversity index of all sequenced breast milk samples.

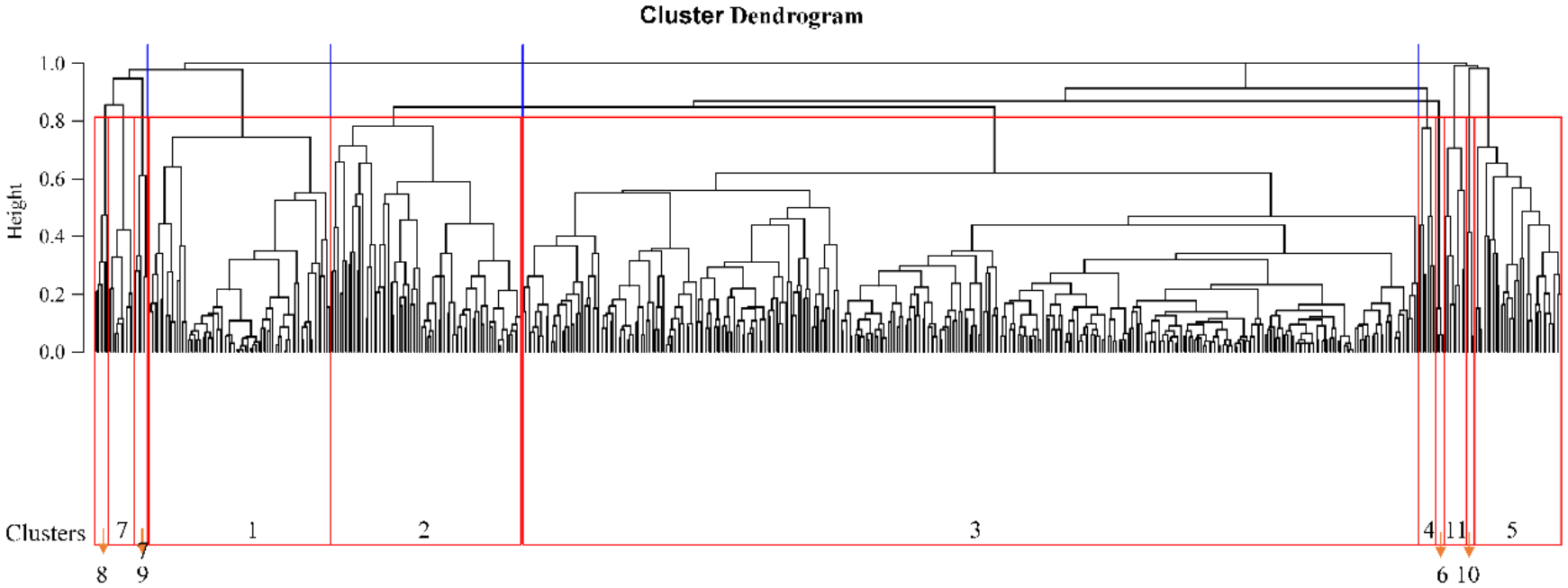


Appendix 2, Figure 3: Bar-plot of relative abundances of bacteria genera. (**A)** Complete linkage unsupervised hierarchical clustering of bacterial abundances within the human breast milk bacteriome at Mbekweni study site (n =235). **(B)** Complete linkage unsupervised hierarchical clustering of bacterial abundances within the human breast milk bacteriome at TC Newman study site (n =287). Each bar represents a mother's HBM bacteriome profile and each coloured box, a bacterial genus. Genera with less than 0.5% abundance in a given sample are grouped together, herein referred to as "other" (grey boxes).

Appendix 2, Table 2: Core taxa in human breast milk bacteriome.

Phylum	Class	Order	Family	Genus	OTU	MRA of taxa in the cohort	Percentage of women with specific taxa (%)
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	OTU_18	0.177	99.8
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Streptococcus*	OTU_20	0.222	99.6
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Streptococcus*	OTU_1	0.262	96.4
Firmicutes	Clostridia	Clostridiales	Veillonellaceae	*Veillonella*	OTU_33	0.015	84.5
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Streptococcus*	OTU_1557	0.002	83.3
Firmicutes	Bacilli	Bacillales	Family_XI_Incertae_Sedis	*Gemella*	OTU_42	0.006	83.3
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Streptococcus*	OTU_2110	0.001	82
Actinobacteria	Actinobacteria	Corynebacteriales	Corynebacteriaceae	*Corynebacterium*	OTU_34	0.012	98.3
Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae	*Rothia*	OTU_38	0.059	94.1
Actinobacteria	Actinobacteria	Corynebacteriales	Corynebacteriaceae	*Corynebacterium*	OTU_30	0.015	92.9
Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae	*Micrococcus*	OTU_41	0.013	92.5
Actinobacteria	Actinobacteria	Corynebacteriales	Corynebacteriaceae	*Corynebacterium*	OTU_113	0.003	84.9
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	OTU_35	0.013	92.1
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unclassified	OTU_24	0.02	86.2

Core taxa in human breast milk bacteriome at different taxonomy levels. Core is defined by the presence of taxa found in HBM from 80% of all mothers enrolled in the study. MRA=Mean relative abundance.



Appendix 2, Figure 4: Dendogram of relative abundances of bacteria in HBM samples at genus level. Hierarchical complete linkage unsupervised clustering on Bray-Curtis dissimilarities shows 11 clusters at a clustering of height 0.8. Cluster 1 (65 members), cluster 2(68 members), cluster 3 (319 members), cluster 4 (6 members), cluster 5 (31 members), cluster 6 (3 members), cluster 7 (9 members), cluster 8 (5 members), cluster 9 (5 members), cluster 10 (3 members), and cluster 11 (8 members).

Appendix 2, Table 3: Mean relative abundance of dominant phyla and genera (>0.5%) across phenotypic clusters in HBM samples.

	Cluster 1	Cluster 2	Cluster 3	p-value
Shannon diversity	1.07	1.65	1.23	< 0.001
Chao1 index	128.84	155.96	174.44	< 0.001
Phylum				
Firmicutes	79.16	68.04	79.62	< 0.001
Actinobacteria	10.93	18.22	14.1	< 0.001
Proteobacteria	9	10.66	5.14	< 0.001
Cyanobacteria	0.43	2.51	0.43	< 0.001
Genus				
Staphylococcus	70.8	32.89	5.62	< 0.001
Streptococcus	6	31.16	68.91	< 0.001
Rothia	0.99	7.24	7.72	< 0.001
Corynebacterium	6.16	5.57	2.45	< 0.001
Acinetobacter	2.17	2.58	1.28	0.0144
Chloroplast	0.4	2.5	0.35	< 0.001
Micrococcus	1.01	1.49	0.73	0.057
OTU_24	2.75	1.43	0.6	< 0.001
Enhydrobacter	0.91	1.42	0.23	< 0.001
Escherichia-Shigella	0.36	1.38	0.3	< 0.001
Lactobacillus	0.1	1.08	0.95	0.101
Bifidobacterium	0.7	0.81	0.71	0.956
Veillonella	0.16	0.71	2.47	< 0.001
Kocuria	0.39	0.52	0.45	< 0.001
Gemella	0.12	0.44	0.74	< 0.001
Pseudomonas	0.47	0.28	0.13	< 0.001

Appendix 2, Table 4: Mean Shannon diversity and mean chao1 index of HBM bacteriome profiles across different socio-economic and psychosocial factors.

	Shannon diversity					
	All samples		Mbekweni clinic		TC Newman clinic	
Covariates	**Mean Shannon diversity**	**p-value**	**Mean Shannon diversity**	**p-value**	**Mean Shannon diversity**	**p-value**
Study site (Ethnicity)		0.535		-		-
Mbekweni	1.343		-		-	
TC Newman	1.3882		-		-	
Mode of delivery		0.337		0.527		0.602
Vaginal	1.3649		1.3287		1.3925	
Elective CS	1.5284		1.5303		1.526	
Emergency CS	1.3219		1.3486		1.2945	
Gestational age		0.492		0.201		0.5

Term	1.3655		1.3713			1.3606		
Pre-term	1.3773		1.2808			1.4565		
Very pre-term	1.3492		1.0337			1.6121		
Infant gender		0.886		0.388			0.386	
Male	1.3697		1.3594			1.3777		
Female	1.3651		1.3243			1.4004		
Infant feeding options		0.575		0.991			0.995	
Exclusive BF	1.3799		1.3434			1.4111		
Mixed BF	1.3519		1.3409			1.3602		
Maternal education		0.236		0.404			0.247	
Primary	1.3724		1.3454			1.3946		
Secondary	1.3661		1.3326			1.3895		
Tertiary	1.3371		1.3587			1.3053		
Maternal employment		0.277		0.459			0.423	
Working	1.4339		1.3209			1.4399		
Not working	1.3468		1.4247			1.3696		
Maternal BMI		0.667		0.443			0.645	
0-18.5	1.333		1.563			1.3089		
18.5-25	1.3521		1.3006			1.3877		
25-30	1.3585		1.4047			1.3138		
30-50	1.4453		1.4138			1.4825		
Infant birth weight	kg	0.346		0.929			0.264	
0-2.5	1.3297		1.3525			1.3176		
2.5-4.5	1.3738		1.395			1.4042		
Infant birth length	cm	0.041*		0.934			0.012*	
0-40	1.4105		1.13			1.7844		
40-48	1.4229		1.4023			1.4343		
48-60	1.3502		1.3365			1.3632		
Maternal age	years	0.036*		0.032*			0.454	
17-22	1.3151		1.2956			1.3306		
22-35	1.3667		1.3106			1.4128		
35-45	1.5333		1.6809			1.3976		
Dwelling type		0.249		0.705			0.329	
Shack	1.3556		1.3156			1.5876		
House	1.3974		1.3653			1.4429		
Flat	1.3076		1.5755			1.3026		
Backyard	1.3378		1.05			1.3648		
Household income		0.807		0.548			0.98	
>R5000/m	1.4479		1.5093			1.4315		
R1000-5000/m	1.377		1.351			1.3948		
<R1000/m	1.33		1.3161			1.3496		
Maternal HIV status		0.379		0.247			0.779	
Negative	1.3594		1.3151			1.3898		
Positive	1.453		1.4737			1.3118		
Household size		0.473		0.133			0.814	
1-5	1.346		1.2882			1.3988		

5-10	1.3951		1.436		1.3667			
10-17	1.5016		1.6911		1.4069			
Maternal smoking		0.644		0.877		0.585		
Non-smoker	1.3395		1.3213		1.3937			
Passive smoker	1.3241		1.318		1.3306			
Active smoker	1.4403		1.4714		1.4341			
Alcohol score		0.948		0.707		0.672		
0-10.5	1.3635		1.3409		1.3829			
10.5-20.5	1.4996		1.0649		1.5589			
20.5-35	1.3106		1.4172		1.2395			
Maternal PTSD		0.384		0.307		0.961		
No exposure	1.3562		1.3404		1.3681			
Trauma exposed	1.2782		1.1243		1.3719			
Suspected PTSD	1.6087		1.5046		1.7607			
Maternal BDI		0.574		0.854		0.902		
Above threshold	1.4243		1.3946		1.4438			
Below threshold	1.3457		1.3293		1.3663			
Maternal SRQ		0.211		0.188		0.428		
Above threshold	1.4483		1.4413		1.4525			
Below threshold	1.3457		1.3208		1.3663			

Appendix 2, Table 4: Mean Shannon diversity mean chao1 index of HBM bacteriome profiles across different socio-economic and psychosocial factors (cont'd).

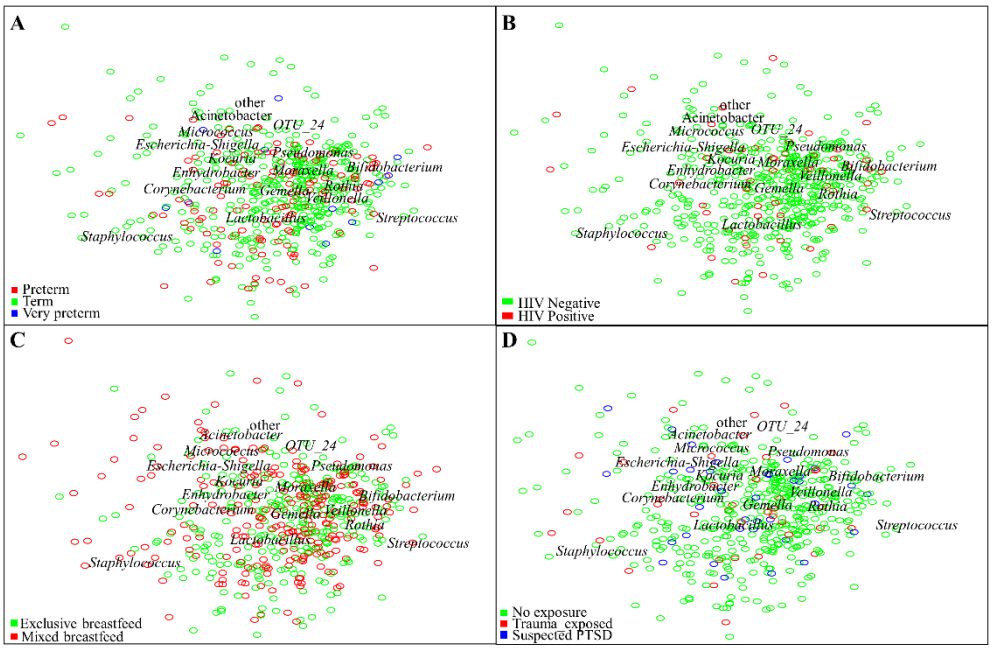
Chao1 index

	All samples		Mbekweni clinic		TC Newman clinic	
Covariates	**Mean Chao1 index**	**p-value**	**Mean Chao1 index**	**p-value**	**Mean Chao1 index**	**p-value**
Study site (Ethnicity)		0.401		-		-
Mbekweni	159.25		-		-	
TC Newman	169.33		-		-	
Mode of delivery		0.971		0.754		0.452
Vaginal	164.22		158.2		168.81	
Elective CS	181.72		166.62		198.82	
Emergency CS	162.11		164.43		159.71	
Gestational age		0.552		0.428		0.415
Term	163.4		157.97		167.85	
Pre-term	170.6		166.11		174.29	
Very pre-term	149.47		128.42		167.02	
Infant gender		0.725		0.036*		0.085
Male	162.34		160.46		163.8	
Female	167.46		157.89		175.74	
Infant feeding options		0.569		0.681		0.906
Exclusive BF	167.01		159.01		173.74	

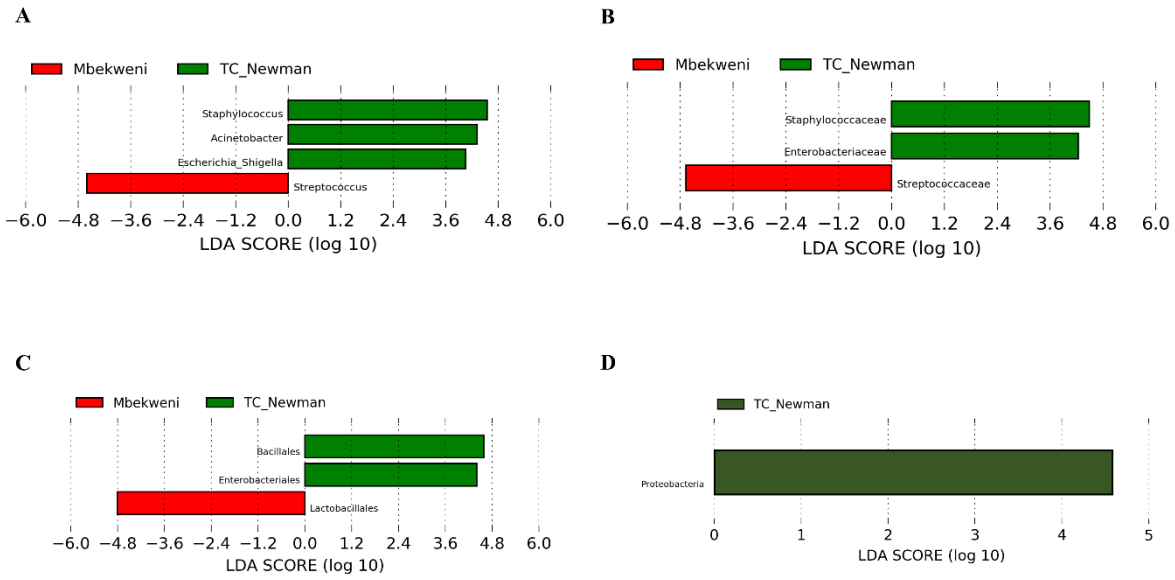
Mixed BF	161.72		158.15		164.44		
Maternal education		0.044*		0.057		0.077	
Primary	172.11		163.41		179.32		
Secondary	153.58		156.98		151.19		
Tertiary	151.51		138.82		170.09		
Maternal employment		0.744		0.863		0.929	
Working	169.12		163.1		173.09		
Not working	163.42		158.21		167.98		
Maternal BMI		0.29		0.776		0.11	
0-18.5	149.33		116.78		152.76		
18.5-25	162.93		162.92		162.95		
25-30	168.5		166.41		170.52		
30-50	172.95		156.52		192.33		
Infant birth weight		0.246		0.689		0.196	
0-2.5	159.11		158.12		159.63		
2.5-4.5	165.86		159.83		171.19		
Infant birth length		0.002**		0.205		0.0009**	
0-40	204.49		196.35		215.36		
40-48	172.16		165.58		175.78		
48-60	161.22		157.59		164.67		
Maternal age		0.63		0.884		0.541	
17-22	156.8		152.38		160.29		
22-35	166.33		159.09		172.28		
35-45	176.21		178.5		174.1		
Dwelling type		0.209		0.881		0.365	
Shack	163.29		157.3		198.04		
House	164.03		158.16		172.36		
Flat	157.45		197.12		156.7		
Backyard	179.72		171.12		180.53		
Marital status		0.891		0.229		0.623	
Living together	170.89		158.39		178.6		
Not living together	160.76		159.62		161.87		
Household income		0.278		0.883		0.115	
>R5000/m	162.26		168.27		160.65		
R1000-5000/m	170.35		157.45		179.13		
<R1000/m	159.22		159.58		158.71		
Maternal HIV status		0.686		0.133		0.345	
Negative	164.74		158.99		168.71		
Positive	165.32		160.47		198.51		
Household size		0.743		0.474		0.604	
1-5	166.01		157.53		173.76		
5-10	161.39		161.64		161.22		
10-17	172.31		175.33		170.79		
Maternal smoking		0.274		0.136		0.762	
Non-smoker	156.78		153.33		167.01		
Passive smoker	162.3		162.12		162.22		

Active smoker	173.44			158.97			170.79	
Alcohol score		0.046*			0.177			0.183
0-10.5	166.36			161.11			170.87	
10.5-20.5	153.01			105.01			159.55	
20.5-35	152.7			136.9			163.24	
Maternal PTSD		0.218			0.096			0.634
No exposure	163.13			155.69			168.74	
Trauma exposed	158.68			154.95			160.88	
Suspected PTSD	189.87			185.66			196.01	
Maternal BDI		0.012*			0.607			0.096
Above threshold	181.08			176.08			184.37	
Below threshold	159.19			153.89			163.65	
Maternal SRQ		0.185			0.839			0.259
Above threshold	165.64			146.57			177.26	
Below threshold	164.49			161.7			166.88	

BMI=Body Mass Index; HIV= Human Immunodeficiency Virus; EBF=Exclusive breastfeeding; PTSD= post-traumatic stress disorder; BDI= Beck Depression Inventory; SRQ= Self-Regulation Questionnaire; CS=Caeserean section. P<0.001, ***; p<0.01, **; p<0.05*.



Appendix 2, Figure 5: Log ratio biplot of human breast milk bacterial abundances at the genus level. Samples are coloured according to **(A)** gestational age, **(B)** maternal smoking, **(C)** Body Mass Index (BMI), and **(D)** delivery mode. Each sample is represented by a circle and is coloured based on group type. Samples (circles) that cluster together are similar in bacteria composition and abundance.



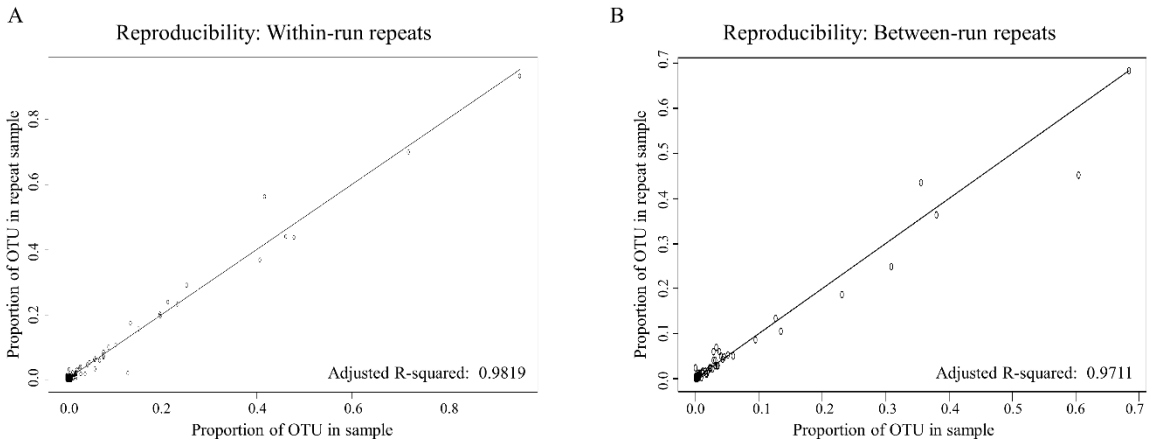
Appendix 2, Figure 6: LEFSe plots showing differences between the HBM bacteriome of participants based on study site. The histograms show the linear discriminant analysis (LDA) scores of bacteriome at **(A)** genus, **(B)** family, **(C)** order and (**D)** phylum level. The higher the LDA score, the more likely it is that the specific taxon is differentially abundant in one group compared to the other group. HBM samples of mothers from Mbekweni study site have higher relative abundance of the taxa shown in red, while HBM samples of mothers from TC Newman study site have higher relative abundance of the taxa shown in green. LEFSe=Linear Discriminant Analysis Effect Size.

Appendix 2, Table 5: Pearson's correlations between the relative abundances of the predominant bacterial genera in the human breast milk samples.

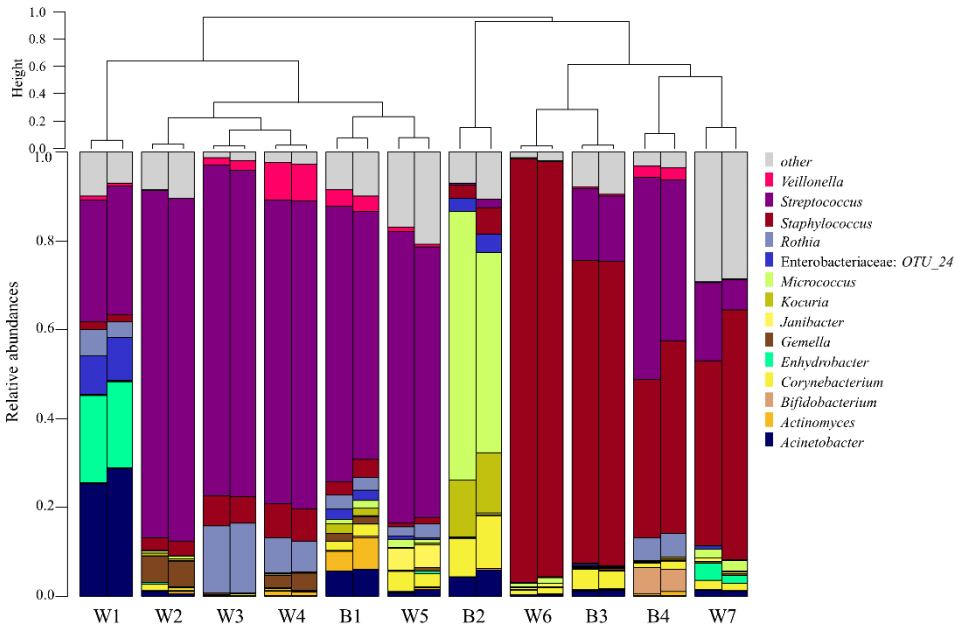
All samples (n=522)

	Acinetobacter	*Bifidobacterium*	*Chloroplast*	*Corynebacterium*	*Enhydrobacter*	*Escherichia-Shigella*	*Gemella*	*Kocuria*	*Lactobacillus*	*Micrococcus*	*OTU_24*	*Pseudomonas*	*Rothia*	*Staphylococcus*	*Streptococcus*	*Veillonella*
Acinetobacter	1															
Bifidobacterium	-0.12	1														
Chloroplast	0.07	-0.09	1													
Corynebacterium	-0.06	-0.08	0.06	1												
Enhydrobacter	0.3	-0.16	0.02	0.01	1											
Escherichia-Shigella	0.07	-0.06	-0.09	-0.15	0.02	1										
Gemella	-0.3	-0.03	-0.1	-0.1	-0.2	-0.25	1									
Kocuria	-0.09	-0.12	-0.11	0.04	-0.04	-0.11	-0.13	1								
Lactobacillus	-0.19	0.01	0	-0.09	-0.19	-0.02	-0.08	-0.07	1							
Micrococcus	0.08	0	-0.17	0.11	0.19	-0.12	-0.09	0.13	-0.12	1						
OTU_24	0.19	-0.15	-0.07	-0.12	0.03	0.42	-0.25	0.01	-0.14	-0.07	1					
Pseudomonas	0.34	-0.19	0.16	-0.09	0.06	0.15	-0.3	-0.12	-0.1	-0.09	0.26	1				
Rothia	-0.25	-0.09	-0.19	-0.21	-0.22	-0.23	0.17	-0.09	-0.14	-0.16	-0.3	-0.26	1			
Staphylococcus	-0.12	-0.11	-0.03	0.4	0.05	-0.04	-0.13	0.07	-0.07	-0.02	-0.02	-0.02	-0.25	1		
Streptococcus	-0.32	-0.01	-0.14	-0.27	-0.32	-0.25	0.39	-0.16	0.12	-0.3	-0.37	-0.28	0.48	-0.33	1	
Veillonella	-0.25	0.05	-0.15	-0.21	-0.27	-0.3	0.26	-0.16	-0.05	-0.18	-0.33	-0.24	0.37	-0.34	0.49	1

Co-occurrences between the relative abundances of the sixteen most predominant bacteria genera. Negative and positive values represent negative and positive correlations respectively between the bacteria. Cut-off value was set to 0.28.



Appendix 2, Figure 7: 16S rRNA gene sequencing reproducibility. Scatter plots represent the relative abundance of each OTU from **(A)** seven within-run repeats and **(B)** four between-run repeats together with their technical repeats. A multiple R-square which is the proportion of variance reproduced by replicating the reads is generated.



Appendix 2, Figure 8: Bar-plot of relative abundances of bacteria genera in human breast milk (HBM) samples and their technical repeats. Complete linkage unsupervised hierarchical clustering of human breast milk bacterial profiles within a sample and its repeat (paired bar-plots). Each bar represents a sample and each coloured box, a bacteria genus. Genera with less than 0.5% abundance in a given sample are grouped together, herein referred to as "other" (grey boxes). W-Within run repeat, B-Between run repeat.

APPENDIX 3: Chapter 6 supplementary tables and figures

Appendix 3, Table 1: Sociodemographic, psychosocial and clinical characteristics of mothers and their infants.

Feature	Participants (n=519) (%)
Socio-demographics characteristics	
Self-defined ethnicity, n (%)	
Black African	229 (44.1%)
Mixed ancestry	290 (55.9%)
Household income, n (%)	
<ZAR1000/m	214 (41.2%)
ZAR1000-5000/m	233 (44.9%)
>ZAR5000/m	72 (13.9%)
Household size, median [95%CI]	5 [2 12]
Maternal characteristics	
Maternal age at enrolment (years), median [95%CI]	25.37 [18.6 39.57]
Maternal BMI, median [95%CI]	25.1 [18.12 40.51]
HIV infected mothers, n (%)	47 (9.1%)
Infant characteristics	
Infant weight at birth (kg), median [95%CI]	3.08 [1.94 3.94]
Infant length at birth (cm), median [95%CI]	50 [41.88 56]
Infant gestational age, n (%)	
Term	403 (77.6%)
Preterm	113 (21.8%)
Very preterm	3 (0.6%)
Infant gender, n (%)	
Female	248 (47.8%)
Male	271 (52.2%)
Pregnancy characteristics	
Exclusive breastfeeding (day), median [95%CI]	55.2 [0 181.75]
Antibiotic during pregnancy, n (%)	7 (1.4%)
Smoking status, n (%)	
Non-smoker	101 (20.4%)
Passive smoker	223 (45.0%)
Active smoker	172 (34.7%)
Alcohol intake during pregnancy, n (%)	67 (14.3%)
HBM sample characteristics	
Collection time (weeks), median [95%CI]	7.43 [5.86 9.43]
Human milk oligosaccharide (HMO) phenotype, n (%)	
Se-/Le-	5 (1.0%)
Se-/Le+	120 (23.1%)
Se+/Le-	84 (16.2%)
Se+/Le+	310 (59.7%)
Lactose concentrations (mM), median [95%CI]	168.16 [80.05 188.49]
Pregnancy characteristics	

IPV Emotional category, n (%)	
No IPV	218 (46.5%)
Isolated incident	64 (13.6%)
Low frequency	38 (8.1%)
Mid frequency	100 (21.3%)
High frequency	49 (10.4%)
Maternal PTSD, n (%)	
No exposure	395 (84.9%)
Suspected PTSD	34 (7.3%)
Trauma exposed	36 (7.7%)
PDI score, median [95%CI]	1.23 [0 3.29]
BDI score, median [95%CI]	11 [0 39]
Maternal BDI Threshold, n (%)	
Above threshold	122 (26.0%)
Below threshold	347 (74.0%)
Maternal SRQ Threshold, n (%)	
Above threshold	102 (21.7%)
Below threshold	368 (78.3%)
Diet characteristics	
Adult food frequency-fish, n (%)	
Never	45 (9.3%)
Monthly	120 (24.8%)
Weekly	256 (53.0%)
Daily	62 (12.8%)
Adult food frequency-starch, n (%)	
Never	0 (0.0%)
Monthly	10 (2.1%)
Weekly	87 (18.0%)
Daily	386 (79.9%)
Adult food frequency- Red meat, n (%)	
Never	81 (16.8%)
Monthly	157 (32.6%)
Weekly	208 (43.2%)
Daily	36 (7.5%)
Adult food frequency-Organ meat, n (%)	
Never	109 (22.7%)
Monthly	173 (36.0%)
Weekly	175 (36.5%)
Daily	23 (4.8%)
Adult food frequency-Poultry, n (%)	
Never	28 (5.8%)
Monthly	20 (4.1%)
Weekly	246 (50.9%)
Daily	189 (39.1%)
Adult food frequency-Eggs, n (%)	
Never	48 (9.9%)

Monthly	58 (12.0%)
Weekly	253 (52.4%)
Daily	124 (25.7%)
Adult food frequency-Processed meat, n (%)	
Never	44 (9.1%)
Monthly	76 (15.8%)
Weekly	247 (51.2%)
Daily	115 (23.9%)
Adult food frequency-Legumes, n (%)	
Never	60 (12.5%)
Monthly	150 (31.2%)
Weekly	230 (47.8%)
Daily	41 (8.5%)
Adult food frequency-Dairy, n (%)	
Never	29 (6.0%)
Monthly	36 (7.5%)
Weekly	172 (35.7%)
Daily	245 (50.8%)
Adult food frequency-Vegetables, n (%)	
Never	16 (3.3%)
Monthly	23 (4.8%)
Weekly	206 (42.7%)
Daily	238 (49.3%)
Adult food frequency-Fruit, n (%)	
Never	13 (2.7%)
Monthly	26 (5.4%)
Weekly	171 (35.4%)
Daily	273 (56.5%)
Adult food frequency-Crisps, n (%)	
Never	34 (7.1%)
Monthly	76 (15.8%)
Weekly	253 (52.5%)
Daily	119 (24.7%)
Adult food frequency-Cold drinks, n (%)	
Never	38 (7.9%)
Monthly	53 (11.0%)
Weekly	209 (43.3%)
Daily	183 (37.9%)
Adult food frequency-Fruit juice., n (%)	
Never	58 (12.0%)
Monthly	125 (25.9%)
Weekly	184 (38.1%)
Daily	116 (24.0%)

BMI=Body Mass Index; HIV= Human Immunodeficiency Virus; EBF=Exclusive breastfeeding; PTSD= post-traumatic stress disorder; IPV= Induced Partner Violence; BDI= Beck Depression Inventory; SRQ= Self-Regulation Questionnaire; Smoking status was measured as Active smoker (cotinine levels ≥ 500); Passive

smoker (cotinine levels 10 – 500); Non-smoker (cotinine levels ≤ 10). Term, Preterm and Very-preterm infants are infant with gestational age of >37 weeks, 32-37 weeks and 28-31.9 weeks respectively.

Appendix 3, Table 2: Comparison of sociodemographic, psychosocial and clinical characteristics between mothers with low and normal breast milk lactose levels.

Feature	Low lactose level (n=45)	Normal lactose level (n=474)	p-value
Socio-demographics characteristics			
Ethnicity, n (%)			9.82E-01
Black African	19 (42.2%)	210 (44.3%)	
Mixed ancestry	26 (57.8%)	264 (55.7%)	
Household income, n (%)			2.42E-01
<ZAR1000/m	23 (51.1%)	191 (40.3%)	
ZAR1000-5000/m	16 (35.6%)	217 (45.8%)	
>ZAR5000/m	6 (13.3%)	66 (13.9%)	
Household size, median [95%CI]	5 [2 11.9]	5 [2 12]	4.77E-01
Maternal clinical characteristics			
Maternal age at enrolment, median [95%CI]	22.98 [18.45 39.02]	25.85 [18.67 39.6]	1.21E-02
Maternal BMI, median [95%CI]	24.8 [17.8 40.88]	25.1 [18.2 39.77]	5.66E-01
HIV infected mothers, n (%)	3 (6.7%)	44 (9.3%)	9.28E-01
Infant clinical characteristics			
Infant weight at birth(kg), median [95%CI]	3.04 [2.33 3.83]	3.1 [1.94 4]	3.95E-01
Infant length at birth(cm), median [95%CI]	49 [43 55.9]	50 [41 56]	1.20E-01
Infant gestational age, n (%)			7.43E-01
Term	34 (75.6%)	369 (77.8%)	
Preterm	11 (24.4%)	102 (21.5%)	
Very preterm	0 (0.0%)	3 (0.6%)	
Infant gender, n (%)			7.41E-01
Female	24 (53.3%)	224 (47.3%)	
Male	21 (46.7%)	250 (52.7%)	
Pregnancy characteristics			
Exclusive breastfeeding (months), median [95%CI]	27.6 [0 150]	55.2 [0 182.4]	2.61E-04
Antibiotic use during pregnancy, n (%)	1 (2.2%)	6 (1.3%)	6.16E-01
Smoking status, n (%)			2.36E-01
Non-smoker	7 (17.1%)	94 (20.7%)	
Passive Smoker	16 (39.0%)	207 (45.5%)	
Active Smoker	18 (43.9%)	154 (33.8%)	
Alcohol exposure, n (%)	8 (19.5%)	59 (13.8%)	5.60E-01

HBM sample characteristics			
Collection time (weeks), median [95%CI]	7.29 [5.6 9.39]	7.43 [5.86 9.43]	1.56E-01
Human milk oligosaccharide (HMO) phenotype, n (%)			5.07E-01
Se-/Le-	1 (2.2%)	4 (0.8%)	
Se-/Le+	10 (22.2%)	110 (23.2%)	
Se+/Le-	3 (6.7%)	81 (17.1%)	
Se+/Le+	31 (68.9%)	279 (58.9%)	
Lactose concentrations (mM), median [95%CI]	97.65 [32.78 126.54]	169.61 [139.38 188.66]	1.36E-28
Pregnancy characteristics			
IPV Emotional category, n (%)			9.03E-01
No IPV	19 (46.3%)	199 (46.5%)	
Isolated incident	6 (14.6%)	58 (13.6%)	
Low frequency	5 (12.2%)	33 (7.7%)	
Mid frequency	6 (14.6%)	94 (22.0%)	
High frequency	5 (12.2%)	44 (10.3%)	
Maternal PTSD, n (%)			7.90E-01
No exposure	34 (82.9%)	361 (85.1%)	
Suspected PTSD	5 (12.2%)	29 (6.8%)	
Trauma exposed	2 (4.9%)	34 (8.0%)	
BDI score, median [95%CI]	16 [0 39]	11 [0 39]	4.51E-02
Maternal BDI Threshold, n (%)			1.20E-02
Above threshold	19 (46.3%)	103 (24.1%)	
Below threshold	22 (53.7%)	325 (75.9%)	
Maternal SRQ Threshold, n (%)			2.55E-01
Above threshold	13 (31.7%)	89 (20.7%)	
Below threshold	28 (68.3%)	340 (79.3%)	
Diet characteristics			
Adult food frequency-fish, n (%)			4.65E-01
Never	4 (9.3%)	41 (9.3%)	
Monthly	8 (18.6%)	112 (25.5%)	
Weekly	25 (58.1%)	231 (52.5%)	
Daily	6 (14.0%)	56 (12.7%)	
Adult food frequency-starch, n (%)			1.85E-01
Never	0 (0.0%)	0 (0.0%)	
Monthly	2 (4.7%)	8 (1.8%)	
Weekly	3 (7.0%)	84 (19.1%)	
Daily	38 (88.4%)	348 (79.1%)	
Adult food frequency-Red meat, n (%)			3.89E-01
Never	4 (9.3%)	77 (17.5%)	

Monthly	16 (37.2%)	141 (32.1%)	
Weekly	19 (44.2%)	189 (43.1%)	
Daily	4 (9.3%)	32 (7.3%)	
Adult food frequency-Organ meat, n (%)			2.01E-02
Never	16 (37.2%)	93 (21.3%)	
Monthly	14 (32.6%)	159 (36.4%)	
Weekly	13 (30.2%)	162 (37.1%)	
Daily	0 (0.0%)	23 (5.3%)	
Adult food frequency-Poultry, n (%)			9.38E-01
Never	3 (7.0%)	25 (5.7%)	
Monthly	1 (2.3%)	19 (4.3%)	
Weekly	22 (51.2%)	224 (50.9%)	
Daily	17 (39.5%)	172 (39.1%)	
Adult food frequency-Eggs, n (%)			6.57E-01
Never	5 (11.6%)	43 (9.8%)	
Monthly	4 (9.3%)	54 (12.3%)	
Weekly	25 (58.1%)	228 (51.8%)	
Daily	9 (20.9%)	115 (26.1%)	
Adult food frequency-Processed meat, n (%)			7.44E-01
Never	1 (2.3%)	43 (9.8%)	
Monthly	10 (23.3%)	66 (15.0%)	
Weekly	21 (48.8%)	226 (51.5%)	
Daily	11 (25.6%)	104 (23.7%)	
Adult food frequency-Legumes, n (%)			3.95E-01
Never	3 (7.0%)	57 (13.0%)	
Monthly	13 (30.2%)	137 (31.3%)	
Weekly	24 (55.8%)	206 (47.0%)	
Daily	3 (7.0%)	38 (8.7%)	
Adult food frequency-Cold drinks, n (%)			2.79E-02
Never	2 (4.7%)	36 (8.2%)	
Monthly	3 (7.0%)	50 (11.4%)	
Weekly	15 (34.9%)	194 (44.1%)	
Daily	23 (53.5%)	160 (36.4%)	
Adult food frequency-Fruit juice., n (%)			2.46E-02
Never	3 (7.0%)	55 (12.5%)	
Monthly	6 (14.0%)	119 (27.0%)	
Weekly	20 (46.5%)	164 (37.3%)	
Daily	14 (32.6%)	102 (23.2%)	

BMI=Body Mass Index; HIV= Human Immunodeficiency Virus; EBF=Exclusive breastfeeding; PTSD= post-traumatic stress disorder; IPV= Induced Partner Violence; BDI= Beck Depression Inventory; SRQ= Self-Regulation Questionnaire; Smoking status was measured as Active smoker (cotinine levels ≥ 500); Passive smoker (cotinine levels 10 – 500); Non-smoker (cotinine levels ≤ 10). Term, Preterm and Very-preterm infants are infant with gestational age of >37 weeks, 32-37 weeks and 28-31.9 weeks respectively.

Appendix 3, Table 3: Comparison of infant anthropometric measures between infants receiving breast milk with low and normal lactose levels.

Features	Low lactose level (n=45)	Normal lactose level (n=474)	p-value
7 weeks			
Z-score for weight, median [95%CI]	-0.46 [-3.74 1.11]	-0.3 [-3.07 1.45]	1.20E-01
Z-score for length, median [95%CI]	-0.74 [-3.35 1.14]	-0.41 [-3.5 1.73]	2.14E-01
Body mass index z-score, median [95%CI]	-0.54 [-2.66 1.57]	-0.13 [-2.45 1.97]	9.64E-02
10 weeks			
Z-score for weight, median [95%CI]	-0.58 [-3.21 1.05]	-0.18 [-2.77 1.87]	1.38E-02
Z-score for length, median [95%CI]	-0.7 [-4.34 2.28]	-0.34 [-3.84 3]	1.61E-01
Body mass index z-score, median [95%CI]	-0.26 [-3.53 1.95]	0.13 [-3.21 2.54]	1.45E-01
14 weeks			
Z-score for weight, median [95%CI]	-0.64 [-3.81 1.31]	-0.13 [-2.34 1.88]	3.68E-03
Z-score for length, median [95%CI]	-1.13 [-3.64 2.47]	-0.3 [-3.59 3.39]	1.95E-02
Body mass index z-score, median [95%CI]	-0.47 [-2.75 2.19]	0.12 [-2.79 2.68]	3.87E-02
6 months			
Z-score for weight, median [95%CI]	-0.04 [-2.23 2.44]	0.08 [-2.65 2.21]	2.67E-01
Z-score for length, median [95%CI]	-0.7 [-3.96 2.62]	-0.07 [-3.18 3.39]	4.85E-02
Body mass index z-score, median [95%CI]	0.38 [-1.59 3.13]	0.32 [-2.41 2.86]	6.57E-01
9 months			
Z-score for weight, median [95%CI]	0.3 [-2.09 2.38]	0.15 [-2.45 2.47]	9.98E-01
Z-score for length, median [95%CI]	-0.35 [-3.36 2.57]	-0.12 [-2.9 2.72]	4.76E-01
Body mass index z-score, median [95%CI]	0.98 [-1.96 2.55]	0.38 [-2.18 2.79]	3.82E-01
12 months			
Z-score for weight, median [95%CI]	0 [-2.55 2.4]	-0.12 [-2.58 2.37]	6.07E-01
Z-score for length, median [95%CI]	-0.1 [-3.15 1.87]	-0.35 [-2.56 1.98]	8.64E-01
Body mass index z-score, median [95%CI]	0.36 [-2.08 2.34]	0.11 [-2.18 2.53]	2.05E-01
18 months			
Z-score for weight, median [95%CI]	0.05 [-2.85 2.16]	0.03 [-2.42 2.42]	7.36E-01

Z-score for length, median [95%CI]	-0.77 [-2.65 1.85]	-0.62 [-3.71 1.93]	8.54E-01
Body mass index z-score, median [95%CI]	0.5 [-2.5 2.54]	0.7 [-2.63 3.74]	4.59E-01
24 months			
Z-score for weight, median [95%CI]	-0.35 [-2.97 2.25]	-0.42 [-2.87 1.95]	9.76E-01
Z-score for length, median [95%CI]	-1.31 [-3.49 0.88]	-1.05 [-3.58 1.34]	2.68E-01
Body mass index z-score, median [95%CI]	0.82 [-1.48 2.94]	0.45 [-1.82 2.97]	2.41E-01

Appendix 3, Table 4: Differences in metabolite profiles of human breast milk from mothers with low and normal lactose levels.

Feature	**Low lactose level (mM)**	**Normal lactose level (mM)**	**p-value**	**FDR**
2-Fucosyllactose, median [95%CI]	3.535 [0 11.824]	4.435 [0 13.788]	7.83E-02	8.90E-02
2-oxoglutarate, median [95%CI]	0.118 [0.048 0.225]	0.113 [0.036 0.374]	4.97E-01	5.18E-01
3-Fucosyllactose, median [95%CI]	0.862 [0 5.789]	1.715 [0 6.276]	8.91E-02	9.90E-02
3-Sialyllactose, median [95%CI]	0.154 [0.048 0.289]	0.093 [0.039 0.183]	6.22E-11	1.20E-10
6-Sialyllactose, median [95%CI]	0.078 [0 0.19]	0.091 [0 0.195]	5.14E-02	5.98E-02
Acetate, median [95%CI]	0.097 [0.04 0.261]	0.047 [0.026 0.103]	7.15E-16	1.88E-15
Acetone, median [95%CI]	0.004 [0.001 0.031]	0.002 [0.001 0.009]	5.66E-07	9.13E-07
Acetyl carnitine, median [95%CI]	0.026 [0.001 0.115]	0.007 [0 0.034]	1.25E-12	2.84E-12
Alanine, median [95%CI]	0.302 [0.093 0.64]	0.302 [0.131 0.598]	6.78E-01	6.92E-01
Asparagine, median [95%CI]	0.025 [0 0.081]	0.034 [0 0.082]	6.30E-03	7.88E-03
Aspartate, median [95%CI]	0.097 [0 0.186]	0.07 [0 0.2]	1.58E-02	1.88E-02
Butyrate, median [95%CI]	0 [0 0.39]	0.203 [0 0.481]	1.62E-10	2.89E-10
Caprate, median [95%CI]	0 [0 0.558]	0.352 [0 0.733]	5.59E-14	1.33E-13
Caprylate, median [95%CI]	0.164 [0 0.627]	0.341 [0 0.617]	1.12E-04	1.56E-04
Choline, median [95%CI]	0.353 [0.073 0.759]	0.197 [0.054 0.675]	4.52E-05	6.46E-05
cis-aconitate, median [95%CI]	0.008 [0 0.04]	0.025 [0.012 0.043]	3.94E-18	1.41E-17
Citrate, median [95%CI]	3.099 [0.685 6.199]	6.025 [3.389 9.345]	8.89E-20	4.04E-19
Creatine, median [95%CI]	0.07 [0.018 0.129]	0.073 [0.026 0.142]	8.12E-01	8.12E-01
Creatinine, median [95%CI]	0.071 [0.036 0.099]	0.052 [0.033 0.081]	8.61E-12	1.79E-11
Cytidine triphosphate, median [95%CI]	0.003 [0 0.044]	0.034 [0 0.101]	1.39E-18	5.35E-18
Cytidine, median [95%CI]	0 [0 0.022]	0.027 [0.005 0.058]	2.13E-21	1.33E-20
Difucosyllactose, median [95%CI]	0.978 [0 8.118]	0.226 [0 1.822]	5.34E-06	8.09E-06
Dimethylamine, median [95%CI]	0 [0 0.004]	0.003 [0 0.007]	5.54E-12	1.20E-11
Formate, median [95%CI]	0.015 [0 0.056]	0.011 [0 0.025]	1.54E-02	1.88E-02
Fumarate, median [95%CI]	0.004 [0.002 0.013]	0.007 [0.002 0.021]	3.35E-06	5.23E-06
Galactose, median [95%CI]	0 [0 0.816]	0 [0 0.77]	4.97E-01	5.18E-01
Glucose, median [95%CI]	0.094 [0 0.383]	0.546 [0 1.109]	2.73E-20	1.52E-19

Glutamate, median [95%CI]	0.69 [0.163 2.038]	1.437 [0.508 2.333]	2.67E-14	6.67E-14
Glutamine, median [95%CI]	0 [0 0]	0.014 [0 0.086]	1.19E-18	4.96E-18
Glycerophosphocholine, median [95%CI]	0.061 [0.015 0.384]	0.613 [0.22 1.079]	2.58E-27	6.45E-26
Isoleucine, median [95%CI]	0.234 [0.052 0.399]	0.041 [0 0.222]	9.14E-22	6.53E-21
Lactate, median [95%CI]	1.606 [0.495 8.114]	0.352 [0.167 1.288]	1.51E-22	1.26E-21
Lactose, median [95%CI]	97.654 [32.783 126.535]	169.608 [139.379 188.661]	1.36E-28	6.80E-27
Leucine, median [95%CI]	0.195 [0.049 0.383]	0.033 [0.011 0.177]	4.95E-24	4.95E-23
LNDFH I, median [95%CI]	2.698 [0 5.886]	0.807 [0 3.685]	8.63E-06	1.27E-05
LNDFH II, median [95%CI]	0.143 [0 1.476]	0.097 [0 0.898]	1.22E-01	1.33E-01
LNFP I, median [95%CI]	2.283 [0 8.4]	1.049 [0 6.563]	4.05E-03	5.19E-03
Lysine, median [95%CI]	0.119 [0.038 0.402]	0.068 [0.008 0.248]	3.33E-07	5.55E-07
Methylguanidine, median [95%CI]	0 [0 0]	0 [0 0.01]	1.06E-03	1.43E-03
Methylhistidine, median [95%CI]	0.05 [0.008 0.102]	0.035 [0.011 0.076]	2.97E-03	3.91E-03
Phenylalanine, median [95%CI]	0.048 [0 0.09]	0.022 [0.01 0.062]	1.23E-09	2.12E-09
Phosphocholine, median [95%CI]	0 [0 0.381]	0.646 [0.033 1.045]	7.04E-25	1.17E-23
Phosphocreatinine, median [95%CI]	0 [0 0.043]	0.035 [0.006 0.09]	3.42E-20	1.71E-19
Pyruvate, median [95%CI]	0.04 [0.017 0.085]	0.022 [0.006 0.055]	1.21E-11	2.42E-11
Succinate, median [95%CI]	0.021 [0.006 0.058]	0.037 [0.015 0.077]	1.54E-10	2.85E-10
Tryptophan, median [95%CI]	0.015 [0.006 0.042]	0 [0 0.016]	1.60E-24	2.00E-23
Tyrosine, median [95%CI]	0.089 [0.025 0.164]	0.031 [0.011 0.114]	1.59E-16	4.68E-16
Uracil, median [95%CI]	0.004 [0 0.072]	0 [0 0.007]	6.29E-17	1.97E-16
Uridine, median [95%CI]	0.078 [0.023 0.197]	0.031 [0.011 0.101]	2.01E-16	5.58E-16
Valine, median [95%CI]	0.236 [0.056 0.411]	0.079 [0.034 0.263]	5.23E-18	1.74E-17

LNDFH II=Lacto-N-difucohexaose II; LNFP I=Lacto-N-fucopentaose I; LNDFH I=Lacto-N-difucohexaose I; FDR: False Discovery Rate

Appendix 3, Table 5: Median relative abundances of predominant human breast milk bacterial genera in mothers with low and normal breast milk lactose levels.

Feature	**Low lactose level (n=44)**	**Normal lactose level (n=441)**	**p-value**	**FDR**
Streptococcus, median [95%CI]	0.32 [0 0.86]	0.58 [0.01 0.87]	1.10E-04	1.32E-03
Staphylococcus, median [95%CI]	0.19 [0 0.98]	0.05 [0 0.84]	4.54E-04	2.00E-03
Rothia, median [95%CI]	0.01 [0 0.18]	0.03 [0 0.35]	1.57E-03	3.77E-03
Corynebacterium, median [95%CI]	0.02 [0 0.38]	0.01 [0 0.27]	5.11E-02	8.76E-02
Veillonella, median [95%CI]	0 [0 0.07]	0.01 [0 0.09]	6.63E-03	1.33E-02
Acinetobacter, median [95%CI]	0 [0 0.12]	0 [0 0.19]	4.57E-01	4.99E-01
Micrococcus, median [95%CI]	0 [0 0.39]	0 [0 0.07]	2.97E-01	3.56E-01
Gemella, median [95%CI]	0 [0 0.01]	0 [0 0.04]	1.56E-03	3.77E-03

Kocuria, median [95%CI]	0 [0 0.01]	0 [0 0.09]	1.01E-01	1.52E-01
Enterobacteriaceae OTU_24, median [95%CI]	0 [0 0.07]	0 [0 0.21]	2.15E-01	2.87E-01
Lactobacillus, median [95%CI]	0 [0 0.01]	0 [0 0.08]	4.99E-04	2.00E-03
Others, median [95%CI]	0.07 [0 0.7]	0.07 [0 0.6]	9.96E-01	9.96E-01

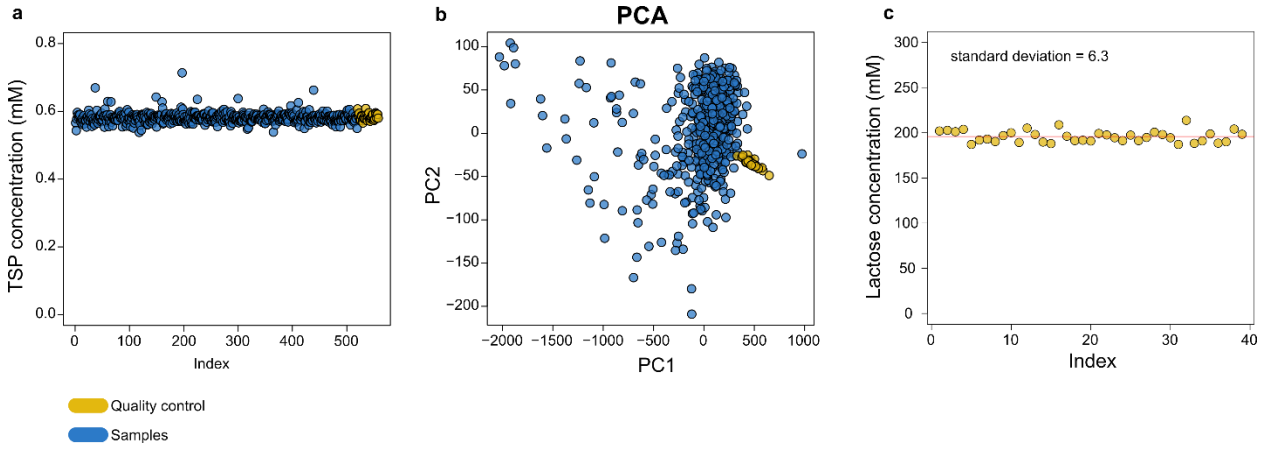
Median values refer to the median relative abundance of bacteria genera. FDR: False Discovery Rate

Supplementary Table 6: List of the metabolites identified in the Nuclear Magnetic Resonance (NMR) spectra.

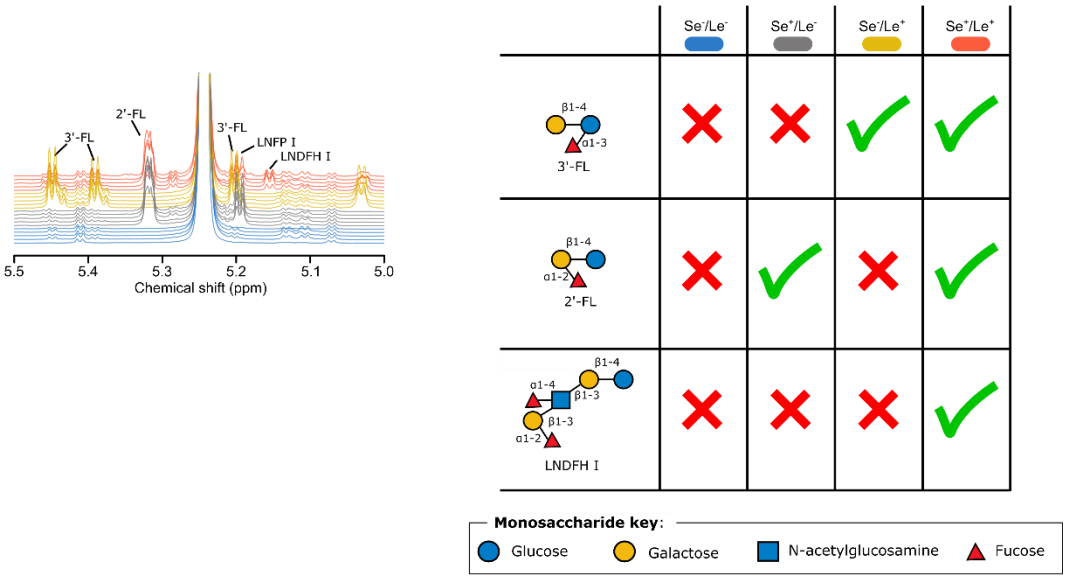
Metabolite	Multiplicity	Number of
2-Fucosyllactose	4.53 (dd), 4.65 (d), 5.32 (d)	0.5
2-oxoglutarate	2.44 (t), 3.01 (t)	2
3-Fucosyllactose	5.20 (d), 5.39 (d), 5.45 (d)	0.5
3-Sialyllactose	2.75 (d), 2.78 (d)	2
6-Sialyllactose	2.71 (d), 2.73 (d)	2
Acetate	1.92 (s)	3
Acetone	2.24 (s)	6
Acetyl carnitine	3.19 (s)	9
Alanine	1.49 (d)	3
Asparagine	2.89 (qd)	2
Aspartate	2.83 (dd)	1
Butyrate	0.90 (t)	3
Caprate	0.88 (t), 2.18 (t)	3
Caprylate	0.87 (t)	3
Choline	3.20 (s)	9
cis-aconitate	3.12 (d), 5.70 (t)	1
Citrate	2.67 (d)	1
Creatine	3.04 (s)	3
Creatinine	3.05 (s)	3
Cytidine	6.08 (d), 7.84 (d)	1
Cytidine triphosphate	6.13 (d), 7.96 (d)	1
Difucosyllactose	5.29 (d), 5.40 (d), 5.46 (d)	0.5
Dimethylamine	2.72 (s)	6
Formate	8.46 (s)	1
Fumarate	6.53 (s)	2
Galactose	4.59 (d)	0.5
Glucose	3.24 (dd), 4.65 (d)	1
Glutamate	2.37 (m)	2
Glutamine	2.48 (m)	2
Glycerophosphocholine	3.23 (s)	9
Isoleucine	0.94 (t), 1.01 (d)	3
Lactate	1.33 (d)	3
Lactose	4.46 (dd), 4.68 (d), 5.25 (d)	0.5
Leucine	0.96 (dd)	6
LNDFH I	2.07 (s), 5.03 (d), 5.16 (d), 8.45 (d)	0.5
LNDFH II	2.05 (s), 5.03 (d), 5.37 (d), 5.43 (d), 8.42 (d)	0.5
LNFP I	2.07 (s), 5.20 (d), 8.41 (d)	0.5
Lysine	3.03 (t)	2
Methylguanidine	2.83 (s)	3
Methylhistidine	7.12 (d), 7.93 (d)	1
Phenylalanine	7.38 (m)	5
Phosphocholine	3.22 (s)	9

Phosphocreatine	3.05 (s)	3
Pyruvate	2.38 (s)	3
Succinate	2.41 (s)	4
Tryptophan	7.74 (d)	1
Tyrosine	7.20 (m)	2
Uracil	5.81 (d)	1
Uridine	7.88 (d)	1
Valine	0.99 (d), 1.04 (d)	3
TSP	0.00 (t)	9

Peak multiplicity: s = singlet, d = doublet, dd = double doublet, t = triplet, h = hextet, m = multiplet. TSP = Trimethylsilylpropionic acid, (internal standard).



Appendix 3, Figure 1: Quality control analysis of the nuclear magnetic resonance (NMR) experiments. a, Trimethylsilylpropionic acid (TSP; internal standard) concentration of the samples and quality controls calculated on the NMR signal at 0.00 ppm. **b,** Principal components analysis (PCA) of the samples and quality controls showing the high reproducibility of the quality controls. **c,** Lactose concentration of the quality control samples.



Appendix 3, Figure 2: Assignment of the Human milk oligosaccharides (HMO) phenotype. The HMO phenotype was associated to each sample according to the presence of 3'FL, LNFP, and LNDFH I signals.